University of Alberta

Characterization of Holliday Resolvase Activity in Herpes Simplex Virus Type 1 Alkaline Nuclease

by

Allan Joseph Ma

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Virology

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Abstract

Replication of herpes simplex virus type 1 (HSV-1) results in the production of a large mass of highly branched DNA. These branches must be removed to release a linear genome for packaging. These branches can be removed by the action of a Holliday resolvase. Therefore, the goal of this thesis was to identify and characterize a Holliday resolvase activity from HSV-1.

The first goal was to determine if HSV-1 infection results in the induction of Holliday resolvase activity because this activity has not previously been described. A partially purified endo-exonuclease from HSV-1 infected cells was able to digest cruciform structures in plasmids that extrude a cruciform from inverted repeats and synthetic cruciforms constructed from oligonucleotides.

The HSV-1 alkaline nuclease (AN) has been suggested as the enzyme responsible for debranching. Partially purified lysates from a null mutant of AN (AN-1) were unable to digest synthetic cruciforms. Since AN-1 expresses all the gene products of HSV-1 except for AN, this suggests AN is involved in the observed Holliday resolvase activity.

To further characterize the role of AN in the Holliday resolvase activity, AN was heterologously expressed in *E. coli* and purified. AN was found to cleave plasmids with inverted repeats without a sequence preference. The cleavage sites were mapped and AN appears to digest at the base of the cruciform in a manner consistent with Holliday resolvases. As well, purified AN could cleave synthetic cruciforms into linear duplex products.

In order to characterize amino acids involved in resolvase activity, site-specific mutagenesis was performed on 5 conserved acidic amino acids. Mutations in 3 of the amino acids (E219, D340, E364) resulted in loss of activity, suggesting that they are

needed for activity. Mutations in E423 resulted in retention of nearly full activity, suggesting that it is not important for catalytic activity. Mutations in D329 resulted in loss of exonuclease activity, but retention of endonuclease activity and Holliday resolvase activity. Therefore, the endonuclease activity of AN is responsible for Holliday resolvase activity.

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Abbreviations and Definitions

A	alanine
AN	alkaline nuclease
AN-1	null mutant of alkaline nuclease gene
ATP	adenosine-5'-triphosphate
dATP	2'-deoxyadensosine-5'-triphosphate
ATPase	adensosine triphosphatase
BSA	bovine serum albumin
bp	base pair
^{o}C	degrees celcius
ccc	covalently closed circular
CHO	Chinese hamster ovary cells
C-terminal	carboxy terminal
CTL	cytotoxic lymphocyte
dCTP	2'-deoxycytidine-5'-triphosphate
D	aspartic asid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DL	delayed late genes
dsDNA	double-stranded DNA
DTT	dithiothreitol
E	early genes
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetic acid
EL	early late genes

ER	endoplasmic reticulum
g	glycoprotein
G	glycine
dGTP	2'-deoxyguanosine-5'-triphosphate
HCF	host cell factor
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HHV-6	human herpesvirus 6
HHV-7	human herpesvirus 7
HHV-8	human herpesvirus 8
hr	hour
Hve	herpesvirus entry mediator
IE	immediate early genes
ICP	infected cell protein
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
K_m	Michaelis constant, substrate concentration at which the reaction rate is half of its maximal value
KSHV	Karposi sarcoma herpesvirus
L	late genes
lacZ	β−galactosidase gene
M	molar
MHC	major histocompatability
min	minute
mg	milligram

µg	microgram
Mg^{2+}	magnesium cation
$MgCl_2$	magnesium chloride
mL	milliliter
µl	microliter
mM	millimolar
Mn^{2+}	manganese cation
$MnCl_2$	manganese chloride
mRNA	messenger RNA
MW	molecular weight
N	asparagine
NaCl	sodium chloride
ng	nanogram
N-terminal	amino terminal
OBP	origin binding protein
oc	open circular
OD_{600}	optical density at wavelength of 600nm
oriL	origin of replication in UL
oriS	origin of replication in US
Pfu	*Pyrococcus furiosis* DNA polymerase
PMSF	phenylmethylsufonyl fluoride
PRR	poliovirus receptor related
PRV	pseudorabies virus
Q	glutamine
RNA	ribonucleic acid
S	serine
SDS	sodium dodecyl sulfate

SN	staphylococcal nuclease
SSC	Salt-Sodium-Citrate
ssDNA	single-stranded DNA
T4	bacteriophage T4
T7	bacteriophage T7
TAP	transporter of antigen presentation
Taq	*Thermus aquaticus* DNA polymerase
TATA box	conserved AT rich region found upstream of the startpoint of each eukaryotic RNA polymerase II transcription unit
TBP	TATA-binding protein
TBE	Tris-Borate-EDTA
TCA	trichloroacetic acid
TFII	transcription factor for RNA polymerase II dependent synthesis of RNA
TK	thymidine kinase
ts	temperature sensitive
TSE	Tris-Salt-EDTA
dTTP	2'-deoxythymidine-5'-triphosphate
UL	unique long
dUMP	2'-deoxyuridine-5'-monophosphate
US	unique short
dUTP	2'-deoxyuridine-5'-triphosphate
vhs	virion host shutoff
VP	viral protein

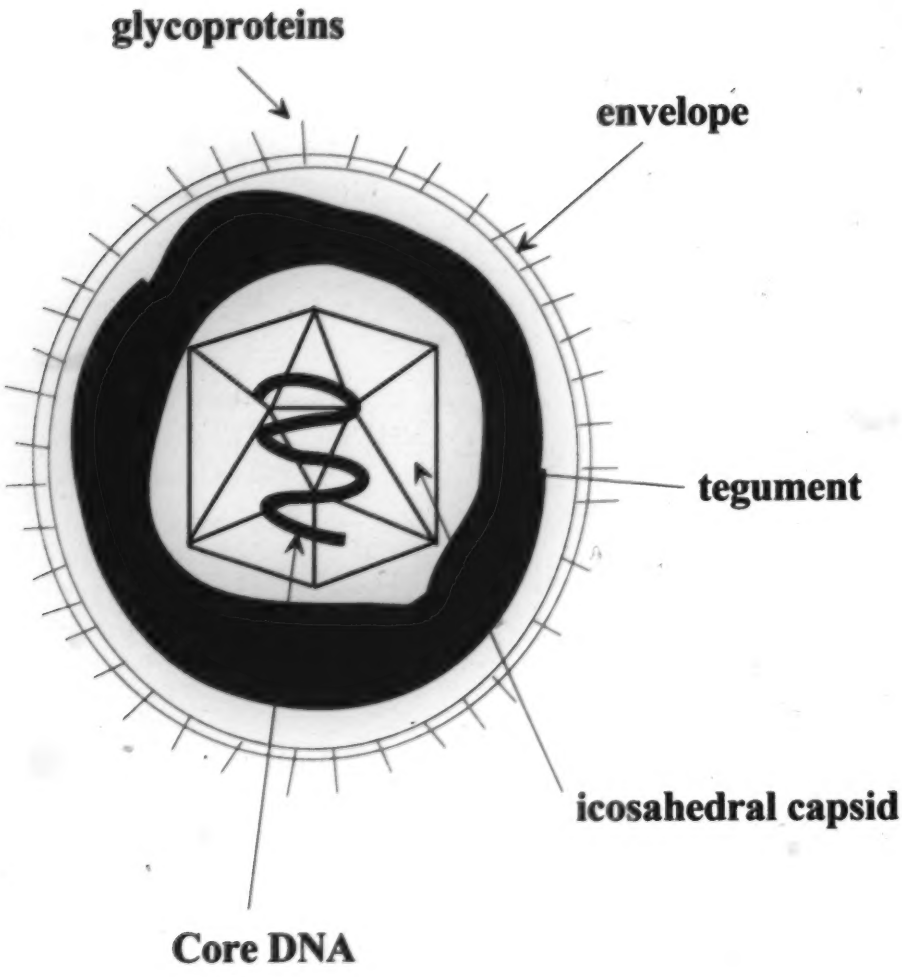
CHAPTER 1
Introduction

1.1 *Herpesviridae* family

The *Herpesviridae* family is comprised of a large class of viruses that are widespread in nature. More than 100 members of the *Herpesviridae* have been identified. They have been isolated from most animal species studied. Inclusion into the *Herpesviridae* family is based on similar structure. A typical virion consists of an electron dense core containing a linear double stranded DNA genome that ranges from 120 to 230 kbp, an icosadeltahedral capsid of approximately 100-110 nm in diameter surrounding the core, an amorphous layer that surrounds the capsid called the tegument, and an envelope containing viral glycoprotein spikes on its surface (Figure 1.1). Herpesviruses synthesize DNA and assemble capsids in the nucleus of an infected cell. The production of infectious progeny virus usually leads to destruction of the cell. However, herpesviruses also enter a latent state after primary infection in which infectious progeny are not produced and only a small subset of viral genes is expressed. Herpesviruses can remain latent for the entire life of the host and the virus can be reactivated in response to the appropriate stimulus (Roizman, 1996, Roizman and Sears, 1996).

Although similar in structure, herpesviruses vary greatly in biological properties such as host range, length of replication cycle, progression of cytopathic events and site of latency. Thus, the herpesviruses have been subdivided into 3 subfamilies (*alphaherpesvirinae, betaherpesvirinae* and *gammaherpesvirinae*) according to biological properties. Members of the *alphaherpesvirinae* subfamily have a wide host range *in vitro*, short reproductive cycle, efficiently destroy infected cells and usually establish latent infections primarily in sensory ganglia. This subfamily is

Figure 1.1 General structure of a herpesvirus. The characteristic structure of herpesvirus virions consists of four elements : (1) a core of linear double stranded DNA; (2) an icosahedral capsid surrounding the core; (3) an amorphous proteinaceous layer called the tegument surrounding the capsid; (4) an outer envelope with glycoprotein spikes on the outer surface.


glycoproteins
envelope
tegument
icosahedral capsid
Core DNA

further divided into two genera. The *Simplexvirus* genus includes the herpes simplex.virus type 1 and type 2 (HSV-1, HSV-2), cercopithecine herpesvirus 1 (herpes B virus), and bovine mammillitis virus. The *Varicellovirus* genus includes varicella-zoster virus (VZV), pseudorabies virus, bovine herpesvirus 1, and equine herpesvirus 1 (Roizman, 1996).

Members of the *betaherpesvirinae* subfamily have a restricted host range, a long reproductive cycle and a latent infection can be established in a wide variety of tissues such as monocytes, epithelial cells, and fibroblasts. Infected cells usually become enlarged (cytomegalia). There are three genera in this subfamily. The *Cytomegalovirus* genus is respresented by human cytomegalovirus (HCMV). The *Muromegalovirus* genus is represented by murine cytomegalovirus. The *Roseolavirus* genus include human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7) (Roizman, 1996).

Members of the *gammaherpesvirinae* subfamily have a narrow host range that is limited to the family or the order of the natural host. They replicate or establish a latent infection in lymphocytes, either T- or B-lymphocytes. In both cases, infectious virus is rarely produced. This subfamily contains two genera. The *Lymphocryptovirus* genus includes Epstein-Barr virus (EBV) as an example. The *Rhadinovirus* genus include herpesvirus ateles and herpesvirus saimiri (Roizman, 1996). The recently identified human herpes virus 8 (HHV-8) or also known as Karposi sarcoma associated herpesvirus (KSHV) is also classified in the *Rhadinovirus* genus (Neipel *et al.*, 1998).

Currently, eight herpesviruses have been isolated from humans. These viruses are herpes simplex virus type 1 (HSV-1 or HHV-1), herpes simplex virus type 2 (HSV-2 or HHV-2), varicella -zoster virus (VZV or HHV-3), Epstein-Barr virus (EBV or HHV-4), human cytomegalovirus (HCMV or HHV-5), human herpes virus type 6

(HHV-6), human herpes virus type 7 (HHV-7), and human herpes virus type 8 (HHV-8 or KSHV). Some of these herpesviruses are clinically important pathogens. Infection with HSV-1 results in cold sores, HSV-2 causes genital herpes, VZV causes chicken pox and shingles upon reactivation from latency, EBV infection can result in infectious mononucleosis and Burkitt's lymphoma, HHV-6 infection can result in a mild disease with a short lived fever called roseola. HCMV and HHV-8 are normally benign in normal individuals but can result in retinitis and Karposi sarcoma, respectively, in immunocompromised individuals such as AIDS patients. There is no clear evidence for involvement of HHV-7 in disease, but it may cause roseola.

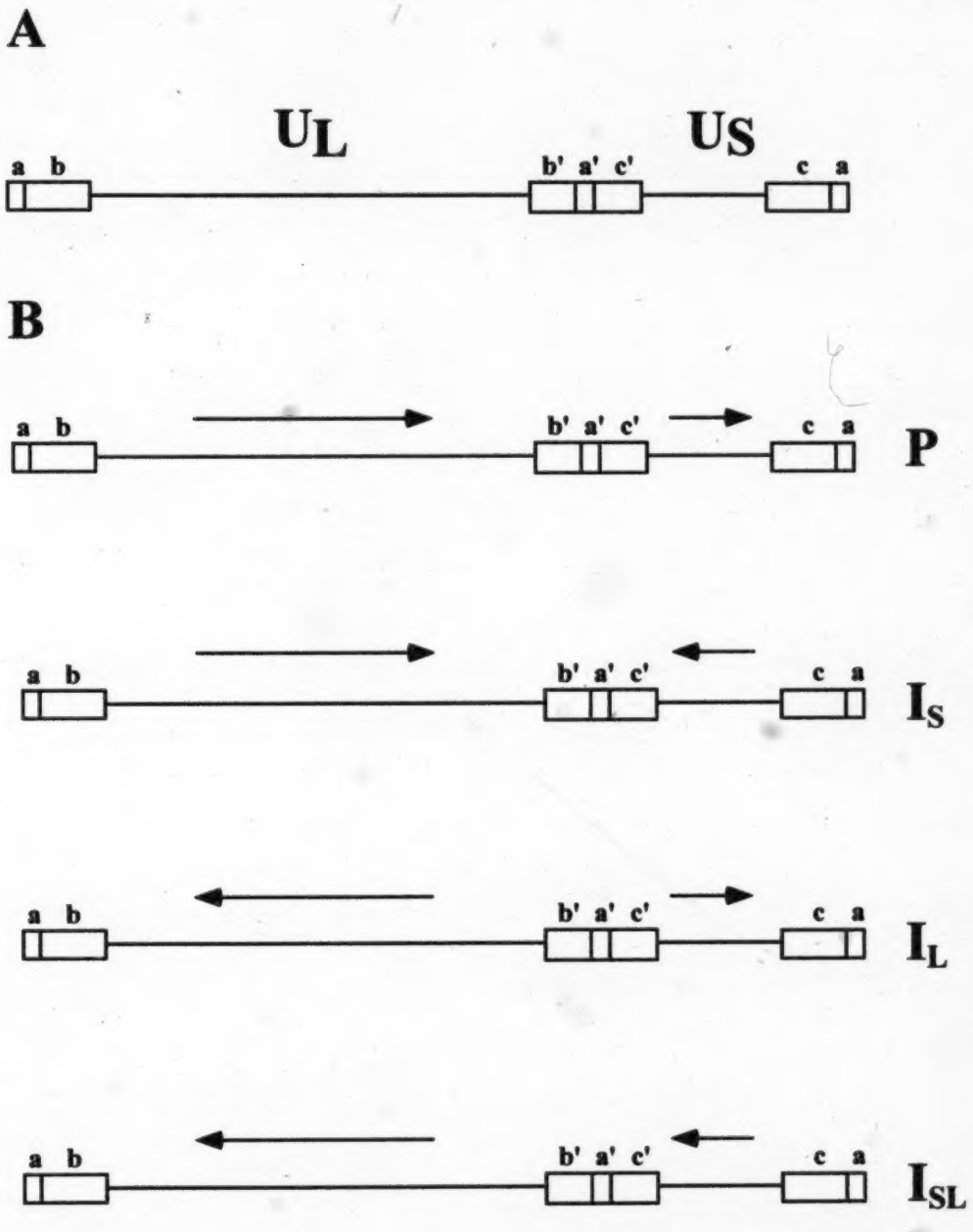
1.2 Herpes Simplex Virus

1.2.1 Clinical features of herpes simplex infection.

HSV infection is initiated by intimate contact at mucosal surfaces in a susceptible, seronegative individual with someone secreting the virus. Viral replication at the site of primary infection ensues which leads to tissue damage (ulceration and vesicular lesions). For HSV-1, infection is usually limited to the oropharyngeal mucosa and to the genital region for HSV-2, although these viruses can cause the reciprocal infection. Virions are then transported by retrograde transport along peripheral sensory neurons to the trigeminal ganglia which becomes colonized. A latent infection is then established which lasts for the life span of the host. In response to stimuli, such as stress, injury or exposure to ultraviolet light, virions are transported anterograde to sites at or near the site of primary infection where a recurrent infection occurs (Whitley, 1996).

Primary infections by HSV-1 usually occur in young children and are most often asymptomatic but can manifest with clinical illness such as gingivostomatitis (primary infection) and herpes labialis (recurrent infection). Infection of the eye, known as keratoconjunctivitis (primary or recurrent infection), may lead to blindness. Primary infection of young adults, however , is usually more severe and can result in pharyngitis and mononucleosis syndrome. HSV-2 is transmitted by sexual contact and primary infection usually does not occur until early adulthood. On rare occasions, the virus can become disseminated causing a severe systemic disease with multiorgan involvement which can be fatal. Infection of the central nervous system leads to encephalitis. Usually infection by HSV is asymptomatic or a mild disease that is self resolving but severe diseases may occur in immunologically immature newborns or

Figure 1.2 Diagram of the herpes simplex virus genome. A. The HSV genome has two components consisting of unique sequences called UL (unique long) and US (unique short). These unique sequences are bracketed by inverted repeats. UL is bracketed by ab and a'b', while US is bracketed by ac and a'c'. B. Inversion of the unique sequences relative to each other during replication results in the generation of equimolar amounts of four isomers. The arrows indicate the relative orientation of the sequences. P is the prototype, I_S indicates inversion of the S component, I_L indicates inversion of the L component, and I_{SL} indicates inversion of both L and S components.


A
UL
US
a b
b' a' c'
c a
B
P
IS
IL
ISL

immunocompromised individuals (Whitley, 1996).

1.2.2 Structure of the virion.

Like all the herpesviruses, the HSV virion consists of four elements: (1) an electron-opaque core containing the viral DNA, (2) an icosadeltahedral capsid surrounding the core, (3) an amorphous proteinaceous layer called the tegument surrounding the capsid, and (4) an outer envelope exhibiting glycoprotein spikes on its surface (Roizman and Sears, 1996) (figure 1.1)

The HSV genome is a large double-stranded linear DNA molecule of approximately 152 kbp with a G+C content of 68% for HSV-1 and 69% for HSV-2 (McGeoch *et al.*, 1985, 1988). The genome consists of two components called L (long) and S (short). The L component consis ts of unique sequences (UL) bracketed by the inverted repeat sequences ab and b'a' while the S component consists of unique sequences (US) bracketed by the inverted repeats ac and a'c' (figure 1.2) (Becker *et al.*, 1968; Kieff *et al.*, 1971; Furlong *et al.*, 1972; McGeoch *et al.*, 1985, 1988). The unique sequences are able to invert relative to each other by homologous recombination between inverted repeats which results in an equimolar mixture of four isomers of the genome that differ in the relative orientation of UL and US (Figure 1.2) (Hayward *et al.*, 1975; Delius and Clements, 1976).

The viral genome has been sequenced and at least 79 genes have been identified : 58 are contained within UL, 13 are within US, 3 within the inverted repeats flanking UL, and 1 in the inverted repeats flanking US (McGeoch *et al.*, 1985, 1988; Roizman and Sears, 1996). In addition, three origins of replication have been identified. There is one origin in UL (oriL) between the UL29 and UL30 genes and two origins in the c

sequences that flank US (oriS). (Stow, 1982; Weller *et al.*, 1985). It is unclear why there are three origins, but all are functional in initiating bi-directional DNA replication.

The capsid of HSV-1 is approximately 120nm in diameter and contains 162 capsomers composed of 150 hexons and 12 pentons (Schrag *et al.*, 1989; Booy *et al.*, 1991; Trus *et al.*, 1992; Newcomb *et al.*, 1993; Zhou *et al.*, 1994). The hexons comprise the capsid edges and faces while pentons are located at each capsid vertex. The capsid is composed of the major capsid protein VP5 (UL19) and three less abundant proteins : VP19C (UL38), VP23 (UL18), and VP26 (UL35) (Gibson and Roizman, 1972; Newcomb *et al.*, 1993). VP5 is the main structural component while VP19C, VP23 and VP26 act in formation and stabilization of the capsid structure (Schrag *et al.*, 1989; Newcomb et al., 1993; Booy *et al.*, 1994; Zhou *et al.*, 1995; Trus *et al.*, 1995, 1996)

The tegument is the amorphous proteinaceous layer between the surface of the capsid and the underside of the envelope. A type of viral particle called L particles are composed of tegument and envelope but lack capsids and core . The existence of L particles demonstrated that the tegument has structural integrity and can self assemble independently of capsids (Szilagyi and Cunningham, 1991, McLauchlan and Rixon, 1992, Zhou *et al.*, 1999). Tegument contains a number of viral proteins such as the transactivator VP16 (UL48), the virion host shutoff (vhs) protein (UL41), VP1/2 (UL36), VP11-12 (UL46), VP13-14 (UL47), VP22(UL49), UL13 protein kinase, and the US11 gene product (Roizman and Spear, 1996). The 4 main structural proteins of the tegument are VP1/2, VP13/14, VP16, and VP22 (Spear and Roizman, 1972; Honess and Roizman, 1973). In addition to their structural role, these proteins provide important functions. VP16 stimulates the expression of immediate early (IE) genes (Post *et al.*, 1981; Batterson and Roizman, 1983; Campbell*et al.*, 1984; Pellett *et al.*, 1985). VP13/14 is thought to be a modulator of VP16-mediated induction of IE genes

(McKnight *et al.*, 1987b; Zhang *et al.*, 1991). VP1/2 is thought to be important in transferring the viral genome from the capsid into the nucleoplasm of an infected cell (Batterson *et al.*, 1983). VP22 is important in transport of capsids due to its interactions with actin microfilaments and ability to stabilize microtubules of the cellular cytoskeleton (Elliot and O'Hare, 1997, 1998). The vhs protein also plays an important role in the life cycle by inducing the degradation of host cell mRNA leading to preferential translation of viral transcripts (Fenwick and Walker, 1978; Fenwick and McMenamin, 1984; Smilbert *et al.* , 1992; Jones *et al.*, 1995) .

Surrounding the structures described above is an envelope derived from host cell membranes. There is some controversy about the subcellular origin of the envelope. One model proposed that after virions acquire envelope by budding from the nucleus, they are transported through the cytoplasm in membrane bound vesicles which fuse with the cellular membrane to release the virions (Johnson and Spear, 1982). The alternative model proposed that enveloped viruses become de-enveloped and re-enveloped in the endoplasmic reticulum or Golgi apparatus during transport (Komuro *et al.*, 1989; Whealy *et al.*, 1991; van Gendersen *et al.*, 1994 ; Browne *et al.*, 1996; Whitley *et al.*, 1999). The viral envelope contains 11 glycoproteins that form the virion spikes (Stannard *et al.*, 1987). These glycoproteins are important for entry into host cell, virion assembly, egress, cell to cell spread, and immune evasion (Rajcani and Vojvodova, 1998). Of the 11 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM) only gB, gC, gD, gH, and gL were shown to be essential for production of virions in cell cultures (Cai *et al.*, 1988; Ligas and Johnson, 1988; Fuller *et al.*, 1989; Forrester *et al.*, 1992; Hutchinson *et al.*, 1992; Roop *et al.*, 1993). There are also nonglycosylated membrane proteins (UL20, UL24, UL34, UL43, UL45, UL49.5) but there is little known about the function of these proteins (Roizman and Sears, 1996).

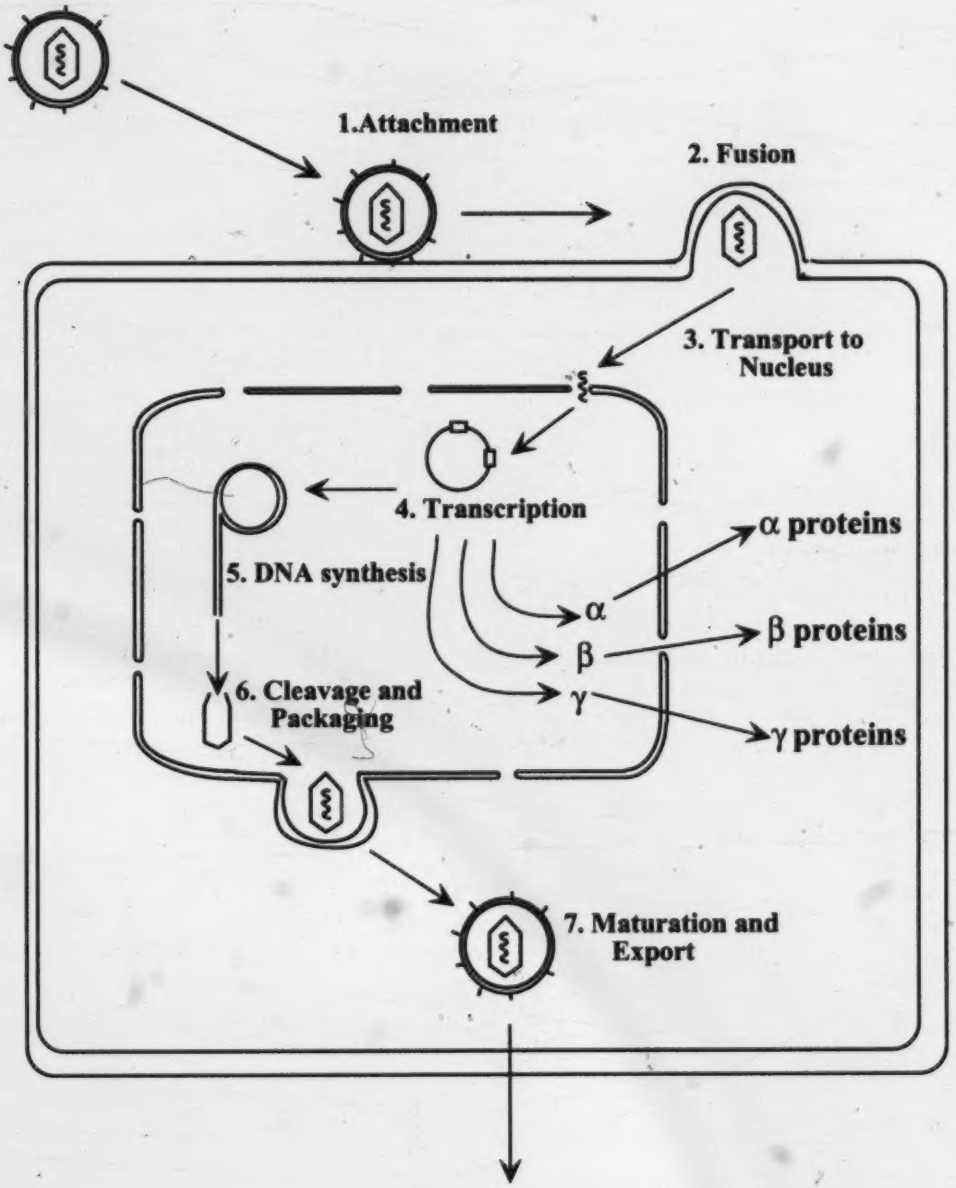
1.3 Herpes Simplex Virus Lytic Life Cycle

In general, lytic infection by HSV is initiated by the attachment of virions to a target cells. Interaction with specific receptors allows fusion of the viral envelope with the plasma membrane releasing the de-enveloped capsid into the cytoplasm of the infected cell. The capsid is transported to the nucleus, the genome is released and circularizes. Viral genes are expressed and the genome is replicated. Finally the viral DNA is packaged into capsids and the progeny virions are released (figure 1.3) (Roizman and Sears, 1996).

1.3.1 Attachment and Entry into Host Cells.

HSV enters cells through at least two sequential steps. There is an initial attachment of virions to the host cell, followed by interaction with specific cellular receptors for penetration. The glycoproteins on the viral envelope are believed to mediate attachment and entry to a target cell. There are 11 glycoproteins encoded by HSV designated as gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM (Stannard *et al.*, 1987; Rajcani and Vojvodova, 1998). Initial attachment was found to be mediated by gC and gB attachment to cell suface heparan sulfate proteoglycan (WuDunn and Spear, 1989; Shieh *et al.*, 1992). Evidence that heparan sulfate served as the receptor for initial attachment was illustrated by the observation that heparin, a soluble molecule that resembles heparan sulfate, inhibited binding of HSV to cells (Spear *et al.*, 1992). Also, the enzymatic removal of heparan sulfate moieties from the cell surface caused a reduction in binding of virions to cells (WuDunn and Spear, 1989). Both gC and gB bound to heparin-sepharose columns (Herold *et al.*, 1991). The binding of virions to cell surfaces was reduced in a gC null mutant and was severely reduced in mutants

Figure 1.3 Lytic life cycle of HSV. 1. Infection of a host cell by a HSV virion is initiated by the attachment to the cell through the interaction of glycoprotein and cell surface heparan sulfate. 2. Interaction with cellular receptors allows penetration of the virion into the host cell via fusion with the cell plasma membrane. 3. The virion is transported to the nucleus by retrograde transport along micotubules to the nuclear pore. Here, the viral DNA is released into the nucleus and the genome circularizes. 4. Expression of genes proceeds through a cascade producing three classes of genes: α (immediate early), β (early), and γ (late). 5. DNA synthesis proceeds through a rolling circle mechanism to produce head-to-tail concatemers. 6. Monomer length genomes are cleaved from the concatemers and packaged into preformed capsids. The packaged capsids egress from the nucleus by budding. 7. Virions mature by acquiring the tegument and glycoproteins and are exported.


1.Attachment
2. Fusion
3. Transport to Nucleus
4. Transcription
5. DNA synthesis
6. Cleavage and Packaging
7. Maturation and Export
α
β
γ
α proteins
β proteins
γ proteins

lacking both gC and gB (Herold *et al.*, 1991, 1994). The initial binding to heparan sulfate is not an absolute requirement for infectivity. Virions were able to attach to cells defective in heparan sulfate biosynthesis via a low efficiency binding to condroitin sulfate (Banfield *et al.*, 1995). Mutants defective in both heparan sulfate and condroitin sulfate retained a low ability to bind HSV and can still be infected (Gruenheid *et al.*, 1993).

HSV enters the target cell by fusion of the viral membrane to the plasma membrane. The virus does not enter through endocytic vesicles as shown by drugs (such as amantadine, chloroquine, and trifluoroperazine) which changed the pH of, and thus inactivated, endocytic vesicles have no effect on HSV entry (Wittels and Spear, 1991). The fusion event requires the participation of four glycoproteins; gD, gB, and the heterodimer gH-gL. Mutants in any of the four glycoproteins produced a virus that could not enter cells (Cai *et al.*, 1988; Ligas and Johnson, 1988; Forrester *et al.*, 1992; Roop *et al.*, 1993). Monoclonal antibodies to each of the four glycoproteins blocked virus infectivity (Pereira *et al.*, 1980; Para *et al.*, 1985; Gompels and Minson, 1986; Navarro *et al.*, 1992; Peng *et al.*, 1998). Of these four glycoproteins, gD has been shown to be the principal glycoprotein capable of binding to cellular receptors. Cells that constitutively expressed gD were resistant to viral infection indicating that the gD sequestered the cellular receptors and made the cells resistant to infection (Campadelli-Fiume *et al.*, 1988; Johnson and Spear, 1989). In addition, preincubation of cells with soluble gD prevented infection (Johnson *et al.*, 1990). However, soluble gC or gH-gL did not block infection (Tal-Singer *et al.*, 1995; Peng *et al.*, 1998). Some viral mutants can infect gD expressing cells. These mutants are referred to as gD unrestricted and contained amino acid substitutions in gD (Dean *et al.*, 1994).

The cellular receptors were identified by transfecting a HeLa cell cDNA library into normally HSV-resistant Chinese hamster ovary (CHO) cells and identifying genes

that mediated infection (Montgomery *et al.*, 1996). The first cellular receptor identified was the herpesvirus entry mediator A (HveA). HveA is a member of the tumor necrosis factor receptor family and is capable of binding gD (Krummenacher *et al.*, 1998). However, HveA expression was limited to T-lymphocytes and was able to mediate entry of wild type HSV-1, but not the gD unrestricted mutants (Montgomery *et al.*, 1996). These results suggested that additional mediators of HSV entry must exist because HSV infects a wide range of cells in culture.

Human receptors that are broadly expressed in human cell lines were identified. They belong to the immunoglobulin G superfamily. This cluster includes three major groups of proteins, each of which has known splice variant isoforms. They are (i) HveC, previously known as PRR1 (poliovirus receptor-related 1) and its splice variant isoform HIgR (herpesvirus immunoglobulin-like receptor) (Geraghty *et al.*, 1998, Cocchi *et al.*, 1998a, 1998b); (ii) HveB, previously known as PRR2α, and PRR2δ and two splice variants (Warner *et al.*, 1998; Lopez *et al.*, 2000); (iii) PVR (the poliovirus receptor), also known as CD155 or HveD, and four known splice variant isoforms (Mendelsohn *et al.*, 1989). All members of this family are transmembrane proteins and share the same overall structure of the ectodomain, made up of one V and two C2 immunoglobulin-type domains. HveC, HveB and PRR2δ function as intercellular adhesion molecules (Lopez *et al.*, 1998; Takahashi *et al.*, 1999; Miyahara *et al.*, 2000). These molecules are recruited at cadherin-based adherens junctions through the binding of their C-terminal conserved motif (A/ExYV) to the PDZ domain of afadin. In turn, afadin binds actin and anchors the adhesion molecules to the cytoskeleton (Takahashi *et al.*, 1999; Miyahara *et al.*, 2000). Because of this activity, HveC, HveB and PRR2δ have been renamed as nectin1δ, nectin2α and nectin2δ, respectively. HIgR does not carry the afadin binding consensus sequence, but has been renamed nectin1α (Lopez *et al.*, 2000; Cocchi *et al.*, 2000)

Nectin1α and -δ expressed in CHO cells render these cells susceptible to infection with all the HSV-1 and -2 strains tested, as well as the animal alphaherpesviruses bovine herpesvirus 1 and pseudorabies virus (Geraghty *et al.*, 1998). Nectin 1α and-δ were found to be expressed in numerous human tissues, including cells of epithelial and neuronal origin (Geraghty *et al.*, 1998). Monoclonal antibodies to nectin1 block infectivity and nectin1 binds to gD (Krummenacher *et al.*, 1998, 1999; Cocchi, 1998a, 1998b). The nectin2 receptors, however, did not serve as receptors for wild type HSV-1 and -2, but do serve as receptors for the gD unrestricted mutants. Cells expressing nectin2 required high multiplicities of infection in order to be infected. Nectin2 binds to gD, but the interaction was weaker than binding to nectin1. Thus, the nectin2 receptors serve as a low efficiency receptor for the unrestricted HSV mutants (Warner *et al.*, 1998; Lopez *et al.*, 2000).

Recently, a new type of molecule was identified as a mediator to virus entry. CHO cells expressing 3-O-sulfated heparan sulphate mediated the entry of HSV-1, but not HSV-2, and 3-O-sulfated heparan sulfate bound to gD (Shukla *et al.*, 1999). In conclusion, there are multiple co-receptors that mediate HSV entry, however, they differ in their specificities.

1.3.2 Transport of Virions to the Nucleus.

After entry into the host cell by fusion, tegument disassembles and the capsid is transported from the cell surface through the cytoplasm to the nuclear pores where the viral DNA is released into the nucleus. The process of tegument diassembly is not well understood. Phosphorylation of the tegument proteins such as VP13/14, VP16, and VP22 lead to dissociation of these proteins from the tegument (Morrison *et al.*, 1998). The dissociation was impaired in virions with inactivated UL13 protein kinase, suggesting that UL13 was involved in tegument diassembly (Morrison *et al.*, 1998).

The transport of HSV-1 capsids is mediated by the cellular microtubule network. Compounds, like colchicine or nocadozole, which depolymerize microtubules, or vinblastine, which causes tubulin paracrystal formation, reduced capsid accumulation at the nuclear envelope (Sodeik *et al.*, 1997). Taxol, which prevents disassembly of microtubules, or cytochalasin D, which causes depolymerization of actin filaments, had no effect on the transport of virions to the nucleus (Sodeik *et al.*, 1997).

The motor used to drive transport of HSV has been identified as cytoplasmic dynein (Sodeik *et al.*, 1997). Cytoplasmic dynein is a multisubunit protein complex consisting of two heavy chains responsible for force production, two to three intermediate chains which are involved in cargo attachment, and a variable number of light chains of unknown function (Holzbaur and Vallee, 1994; Schroer, 1994). Dynein is responsible for retrograde transport of organelles and chromosomes along microtubules. The intermediate chain of dynein was found to interact with the HSV protein UL34 (Ye *et al.*, 2000). UL34 protein is a membrane associated protein that is a substrate for US3 protein kinase (Purves *et al.*, 1992). It is believed that after entry into cells, UL34 becomes exposed, interacts with the dynein motor, and uses the microtubular network for retrograde transport of capsid to the nucleus (Ye *et al.*, 2000).

Release of the viral DNA from the capsid appears to be mediated by the tegument protein VP1/2. Capsids of the temperature sensitive mutant HSV-1(HFEM)tsB7 accumulate at nuclear pores and only release viral DNA after a shift down from nonpermissive to permissive temperatures (Batterson *et al.*, 1983). The mutation in HSV-1(HFEM)tsB7 was mapped to UL36 which encodes VP1/2 (Batterson *et al.*, 1983; McNabb and Courtney, 1992a, 1992b).

1.3.3 Viral Gene Expression.

The expression of viral genes proceeds through a highly regulated cascade involving the sequential expression of three kinetic classes of genes : immediate early (IE or α), early (E or β), and late (L or γ) (Honess and Roizman, 1974). In general, IE genes encode regulatory proteins required for induction and maintenance of E and L gene expression and inhibition of IE gene expression. The E genes encode for proteins involved in viral DNA replication. The L gene products are mostly structural proteins. Transcription of HSV genes is performed by host cell RNA polymerase II complexes (Preston and Newton, 1976; Beck and Millette, 1982; Rice *et al.*, 1994).

Immediately upon entry of the virion into a cell, a virion tegument protein called vhs (virion host shutoff) causes the shutoff of host protein synthesis and accelerated mRNA degradation (Fenwick and Walker, 1978; Fenwick and Mcmenamin, 1984; Smilbert *et al.*, 1992; Jones *et al.*, 1995). Vhs was found to inhibit reporter gene expression when transiently expressed in mammalian cells (Jones *et al.*, 1995; Pak *et al.*, 1995) and trigger the accelerated degradation of reporter RNAs when it was produced in a rabbit reticulocyte lysate system (Zelus *et al.*, 1996; Elgadi *et al.*, 1999). Thus, vhs can induce the shutoff of protein synthesis in the absence of other viral proteins. Vhs was shown to act as an endoribonuclease but no specificity was detected (Elgadi *et al.*, 1999). However, the insertion of a picornaviral internal ribsosome entry site (IRES) into an RNA substrate directed vhs endoribonucleolytic cleavage to sequences immediately 3' to the IRES suggesting that vhs may be recruited to a target mRNA though the interaction with translation initiation factors or that the IRES forms a unique secondary structure that vhs recognizes (Elgadi and Smiley, 1999; Lu *et al.*, 2001). The shutoff of cellular protein synthesis allows the diversion of the cellular synthetic machinery to the production of viral components.

Another tegument protein, the VP16 transactivator, is responsible for stimulating IE gene expression (Post *et al.*, 1981; Batterson and Roizman, 1983; Campbell *et al.*, 1984; Pellett *et al.*, 1985). All IE gene promoters have the consensus sequence 5'- GyATGnTAATGArATT - 3', also known as TAATGARAT, where R is a purine (Mackem and Roizman, 1982a, 1982b). Although the TAATGARAT sequence is necessary for IE gene transactivation (Preston *et al.*, 1984), VP16 alone interacted weakly with this sequence (Kristie *et al.*, 1989; Kristie and Sharp, 1990). Recognition of this sequence was greatly enhanced upon formation of a VP16-cellular protein complex (Kristie and Roizman, 1987; McKnight *et al.*, 1987a; O'Hare and Goding, 1988; O'Hare *et al.*, 1988). VP16 first binds to the cellular protein HCF (host cell factor), a nuclear factor involved in cell proliferation (Xiao and Capone, 1990; Wilson *et al.*, 1993, 1997; Kristie *et al.*, 1995; Goto *et al.*, 1997; LaBoissiere *et al.*, 1997, 1999; Johnson *et al.*, 1999; Liu *et al.*, 1999;). Co-expression of HCF and VP16 resulted in the nuclear accumulation of VP16 (LaBoissiere *et al.*, 1999). Since VP16 lacks a nuclear localization signal, HCF was proposed to act as a nuclear importer of VP16 (LaBoissiere *et al.*, 1999). VP16 and HCF together associate with Oct-1, a transcription factor (Gerster and Roeder, 1988; Kristie *et al.*, 1989; Stern *et al.*, 1989; Kristie and Sharp, 1990; Stern and Herr, 1991). Once targeted to HSV IE promoters through association with HCF and Oct-1, VP16 activates transcription through its highly acidic carboxy terminal 80 amino acids (Sadowski *et al.*, 1988; Trizenberg *et al.*, 1988; Cousens *et al.* , 1989). A chimeric protein of the C-terminal domain fused to the yeast transcriptional activator GAL4 DNA binding domain induced transcription of a reporter gene to a high level (Sadowski *et al.*, 1988; Trizenberg *et al.*, 1988; Cousens *et al.*, 1989). The mechanism by which VP16 induces transcriptional activation is not clear but the C-terminal domain was shown to interact with TFIIB, the TATA-box binding subunit of TFIID, and TFIIH of the RNA polymerase II holoenzyme (Stringer

et al., 1990; Ingles *et al.*, 1991; Lin *et al.*., 1991; Goodrich *et al.*, 1993; Xiao *et al.*, 1994; Gupta *et al.*, 1996).

Vhs accelerated the degradation of both viral and cellular mRNA (Kwong and Frenkel, 1987). VP16 was found to bind to vhs and this interaction may modulate vhs activity (Smilbert *et al.*, 1994; Lam *et al.*, 1996). Consistent with this is the finding that a VP16 null mutant exhibited greatly exaggerated mRNA turnover and essentially complete translation arrest at intermediate times postinfection (Lam *et al.*, 1996). VP16 may downregulate vhs activity at intermediate and late times postinfection to allow maintenance of viral protein synthesis.

1.3.4 Immediate Early Genes.

Five IE genes are efficiently expressed early in infection to VP16 transactivation : infected cell protein 0 (ICP0), ICP4, ICP22, ICP27, and ICP47 (Roizman and.Sears, 1996). All the IE genes, except for ICP47, are involved in gene regulation. ICP47 is able to inhibit the presentation of viral antigens on the surface of infected cells thereby evading recognition by CD8+ cytotoxic lymphocytes (CTL) (York *et al.*, 1994; Fruh *et al.*, 1995; Hill *et al.*, 1995; Ahn *et al.*, 1996; Tomazin *et al.*, 1996, Lacaille and Androlewicz, 1998). The antigen presentation pathway requires intracellular degradation of the antigenic protein to peptide fragments, transport of these peptides to the endoplasmic reticulum (ER) by the heterodimeric transporter of antigen presentation (TAP), assembly of class I major histocompatability complex (MHC) heavy chain , β2-microglobulin, and peptide complexes within the ER lumen, and transport of this trimeric complex to the plasma membrane where it is displayed for recognition by CTL (Hemmels and Ploegh, 1995). ICP47 bound tightly to the peptide binding site of TAP and prevented the binding of other peptides that were suitable substrates for TAP leading to the shutdown of the TAP-dependent flow of peptides into the ER (Ahn *et al.*,

1996; Tomazin *et al.*, 1996). As well, ICP47 destabilizes the TAP heterodimer by inducing a loose association of the subunits thereby contributing to inhibition of TAP function (Lacaille and Androlewicz, 1998).

ICP4 is a major regulatory phosphoprotein in HSV. During viral infection, ICP4 is required for the transcriptional activation of most of the essential E and L genes (Watson and Clements, 1980). ICP4 deletion or temperature sensitive mutants are non-viable due to defects in expression of E and L genes (Watson and Clements, 1978; Preston, 1979; Dixon and Schaffer, 1980; DeLuca and Schaffer, 1985; DeLuca *et al.*, 1985). ICP4 also acts as a repressor of its own expression (Dixon and Schaffer, 1980, DeLuca *et al.*, 1985; Roberts *et al.*, 1988; Michael and Roizman, 1989). The DNA binding activity of ICP4 is important for its role as a transcription activator and repressor since mutations in the ICP4 DNA binding domain resulted in loss of transcription regulation function (Paterson and Everett, 1988; Shepard and DeLuca, 1989, 1991) . A consensus DNA binding site has been identified (Faber and Wilcox, 1986; Michael *et al.*, 1988). However, a large number of ICP4 responsive genes lack this sequence indicating that ICP4 could bind at other sequences or act through other mechanisms (Imbalzano *et al.*, 1990; Smiley and Duncan, 1992; Smiley *et al.*, 1992; Gu and DeLuca, 1994). ICP4 has been shown to form a tripartite complex on DNA with the TATA-binding protein (TBP) and TFIIB and facilitate the binding of TFIID to the TATA box in the presence of TFIIB (Smith *et al.*, 1993). This complex has been shown to mediate repression (Gu *et al.*, 1995). ICP4 also can interact with TFIID , through interaction of ICP4 and TAF250, to activate transcription (Carozza and DeLuca, 1996). Other cellular proteins may be involved, such as HMG-1 and NFκB, but their roles are not clear (Patel *et al.*, 1998; Panagiotidis and Silverstein, 1999).

ICP27 is an essential protein required for E and L gene expression. ICP27 does not act at the transcriptional level. It has been shown to act at a post-

transcriptional level by inhibiting pre-mRNA splicing, which may provide an advantage to the virus whose mRNAs are predominately unspliced (Sandri-Goldin and Mendoza, 1992; Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994). As well, ICP27 was able to enhance the expression of genes with weak 3' polyadenylation signals by stabilizing the 3' end which increased the 3' polyadenylation (Sandri-Goldin and Mendoza, 1992; Brown *et al.*, 1995; McGregor *et al.*, 1996). Late mRNAs from HSV are known to contain weak 3' processing signals (McGregor *et al.*. 1996). Also, ICP27 was shown to shuttle between the nucleus and cytoplasm of an infected cell (Phelan and Clements, 1997, Soliman *et al.*, 1997). ICP27 binds intronless mRNA in the nucleus causing a conformational change in ICP27 to unmask its nuclear export signal and allow export to the cytoplasm (Soliman and Silverstein, 2000). Therefore, ICP27 is able to reduce the amount of competing cellular mRNA by increasing the levels of unspliced 3' poly(A) viral mRNA in the cytoplasm of an infected cell.

ICP0 is a promiscuous transactivator that activates the expression of any viral or cellular gene (Everett, 1984; O'Hare and Hayward, 1985, Cai and Schaffer, 1992). Infection at a low multiplicity of cells with a mutants lacking functional ICP0 resulted in the lack of activation of viral transcription and establishment of a quiescent infection which was refractory to activation by VP16 or ICP4 (Stow and Stow, 1986; Everett, 1989, Cai and Schaeffer, 1991). However, provision of exogenous ICP0 allowed for reactivation of the quiescent genomes and entry into the lytic cycle (Harris *et al.*, 1989; Stow and Stow, 1989; Preston and Nicholl, 1997; Samaniego *et al.*, 1998). ICP0 was found to localize to the nucleus to nuclear substructures called ND10, PML nuclear bodies or PODs (PML oncogenic domains) and during infection, ICP0 completely disrupts ND10s (reviewed in Everett, 2000a). There are usually 5 to 20 ND10s per cell nucleus but the function of these structures is unknown (Maul, 1998). ICP0 causes similar disruption of centromeres by degradation of the essential centromere

protein CENP-C. ICP0 was found to interact with the ubiquitin specific protease USP7. In the ubiquitin-proteosome pathway of protein degradation, ubiquitin chains are conjugated to a target protein which is then recognized and degraded by the proteosome. The ubiquitin specific protease cleaves the bond between ubiquitin and the target protein, thus protecting the target protein from degradation (Wilkinson, 1995). ICP0 may eliminate the protective ability of USP7 leading to changes in protein stability. USP7 was found to accumulate in or near ND10. The ubiquitin-like protein SUMO-1 was also found in ND10. The ND10 proteins PML and Sp100 were covalently modified by SUMO-1, leading to the disruption of ND10. ICP0 can induce the proteosome-dependent degradation of PML and Sp100 and this activity is directly related to the ability of ICP0 to disrupt ND10. These findings led to the hypothesis that ICP0 stimulates viral infection by inducing the proteosome-dependent degradation of cellular proteins and that one or more of the target proteins could be involved in a repression mechanism that would otherwise repress viral transcription (Everett, 2000a, 2000b).

The ICP22 protein is dispensable for growth, but this appears to be cell specific. In Vero and HEp-2 cells, replication of ICP22 mutants is as efficient as wild type. whereas in BHK, RAT-1, and HEL cells, ICP22 mutants grew poorly (Post and Roizman, 1981; Sears *et al.*, 1985; Poffenberger *et al.*, 1993; Purves *et al.*, 1993). ICP22 was believed to act at the transcriptional level. HSV infection caused an alteration in the phosphorylation status of RNA polymerase II (Rice *et al.*, 1994). Hypophosphorylated (IIa) and hyperphosphorylated (IIo) forms of RNA polymerase II were depleted and a novel intermediately phosphorylated (IIi) form accumulated (Rice *et al.*, 1994). This accumulation of the IIi form of RNA polymerase II was dependent on functional ICP22 and UL13 protein kinase (Rice *et al.*, 1995; Long *et al.*, 1999). The IIi form was observed to be the most transcriptionally active form of RNA

polymerase II by nuclear run-on transcription analysis in infected cells (Spencer *et al.*, 1997). This modification favored the transcription of viral mRNA because repression of host cell transcription is correlated with the depletion of the IIo form of RNA polymerase II (Spencer *et al.*, 1997).

In addition to the individual roles of ICP4, ICP27, and ICP0 on regulation of gene expression, it has been shown that they are able to interact with each other. ICP0 and ICP4 were shown to act synergistically such that gene expression was greater when they were co-expressed than observed when either protein was expressed alone. Consistent with this observation, ICP0 and ICP4 were shown to physically interact with each other (Yao and Schaffer, 1994). Also, a physical and functional interaction between ICP4 and ICP27 has been reported (Panagiotidis *et al.*, 1997). It was found that ICP27 can modulate the ability of ICP4 to form a stable DNA-protein complex on a noncanonical binding site present in the HSV thymidine kinase gene but not the canonical site from the ICP4 gene (Panagiotidis *et al.*, 1997). Thus, the IE proteins regulate gene expression through a complex process of protein-protein interactions.

1.3.5 Early genes and DNA Replication.

Replication of the HSV genome occurs in the nucleus of an infected cell. As stated previously, there are 3 origins of replication in the HSV genome : 2 copies in the c sequences of the inverted repeats flanking US (oriS) and one between UL29 and UL30 (oriL) (Stow, 1982; Weller *et al.*, 1985). Both oriS and oriL contain large palindromes of 45 and 144 bp, respectively, that are centered around an A+T rich region of 18 and 20 bp, respectively. Flanking the A+T rich regions are binding sites of the UL9 origin binding protein (discussed below). It is unclear why there are multiple origins that are functionally similar. Deletion of either one or both copies of

oriS or oriL has no effect on viral DNA replication in cultured cells (Longnecker and Roizman, 1986; Polvino-Bodnar *et al.*, 1987; Igarashi *et al.*, 1993). In transient assays, both types of origins replicate with similar kinetics and to similar extents (Hardwicke and Schaffer, 1995). It remains unclear why these multiple origins are conserved.

After entry into the nucleus of an infected cell, the genome circularizes and acts as the template for origin-dependent bi-directional DNA replication. Endless forms of newly replicated DNA are produced until the the very late stages of replication, presumably as a result of a rolling circle mechanism of DNA replication (Jacob and Roizman, 1977, Becker *et al.*, 1978; Jacob *et al.*, 1979; Poffenberger and Roizman, 1985). The fact that replicating DNA consists of rapidly sedimenting DNA in sucrose gradients with S values greater than that of mature viral DNA (Jacob *et al.*, 1979) is evidence supporting the rolling circle model. Replication of plasmids containing a HSV origin in cells co-infected with HSV-1 produced large head-to-tail concatemers (Stow, 1982). Digestion of replicating DNA by restriction enzymes that digest once per genome yields genome length product demonstrating the concatemeric arrangement of replication intermediates (Severini *et al.*, 1994; Zhang *et al.*, 1994). Also, extracts from insect cells infected with recombinant baculoviruses encoding the HSV-1 enzymes required for DNA replication or extracts prepared from HSV-1 infected human cells promoted rolling circle replication of a circular plasmid that lacked a HSV origin (Skaliter and Lehman, 1994; Skaliter *et al.*, 1996). However, the structure of replicating DNA has been shown to be highly complex. Electron microscopic examination of replicating DNA has revealed that the DNA contains extensive regions of single-stranded DNA, DNA replication forks, and branched DNA structures (Shlomai *et al.*, 1976; Friedman *et al.*, 1977; Severini *et al.*, 1996). Pulsed-field gel analysis demonstrated that after digestion of replicating DNA with a restriction enzyme

that cuts once per genome, most of the DNA remains in the well of the gel, suggesting that the DNA was branched (Severini *et al.*, 1994; Zhang *et al.*, 1994). Direct evidence for these branched structures in the complex viral DNA was obtained by two-dimensional gel electrophoresis and electron microscopy (Severini *et al.*, 1996). It is not known how the branches form during replication, however recombination is tightly coupled to replication (Weber *et al.*, 1988; Dutch *et al.*, 1995; Ukeme, 1999). The UL and US segments are able to invert relative to each other via homologous recombination resulting in equimolar amounts of each of the isomers (fig 1.2), which demonstrates the involvement of recombination during replication (Hayward *et al.*, 1975; Delius and Clements, 1976; Davison and Wilke, 1983; Weber *et al.*, 1988; Sarisky and Weber, 1994; Martin and Weber, 1996).

The products of early (E) genes are generally involved in viral DNA synthesis. Seven of the E gene products have been identified as necessary and sufficient for DNA synthesis. These proteins were identified by their ability to replicate a plasmid containing a HSV origin of replication in transient transfection of mammalian cells or when expressed in insect cells using recombinant baculovirus (Challberg, 1986; Wu *et al.*, 1988; Stow, 1992). The genes identified were UL30 (DNA polymerase), UL42 (DNA polymerase processivity factor), UL9 (origin binding protein), UL29 (single-stranded DNA binding protein), and UL5, UL8, and UL52 (DNA helicase-primase). Mutations in any one of these genes abrogated viral replication (Coen *et al.*, 1984; Carmichael and Weller, 1989; Johnson *et al.*, 1991; Malik *et al.*, 1992; Zhu and Weller, 1992; Klinedinst and Challberg, 1994).

The DNA polymerase holoenzyme exists as a heterodimer of UL30 and UL42 proteins. The UL30 subunit is a 1235 amino acid, 136kDa protein (Boehmer and Lehman, 1997) that exhibits significant sequence similarity to other viral and cellular DNA polymerases such as human DNA polymerase α-primase, *Saccharomyces*

cerevisiae DNA polymerase δ, *E. coli* DNA polymerase I, and bacteriophage T4 DNA polymerase (Digard and Coen, 1990; Blanco *et al.*, 1991). The domains responsible for DNA polymerization were determined by demonstrating that deletion of residues 881-959 or amino acid substitutions within this region abolished DNA polymerase activity (Dorsky and Crumpacker, 1988; Knopf and Weisshart, 1988). In addition to the DNA polymerization activity, the UL30 subunit possesses a 3'-5' exonuclease (Knopf, 1979; Marcy *et al.*, 1990; Hall *et al.*, 1996). In the presence of the 4 dNTPs there was no exonuclease activity on 3'-paired deoxynucleotides, whereas 3'-unpaired deoxynucleotides were completely removed (O'Donnell *et al.*, 1987; Abbotts *et al.*, 1987). This showed that the 3'-5' exonuclease serves in proofreading to ensure high fidelity of DNA replication (Abbotts *et al.*, 1987). The UL30 contains 3 sequence motifs that are homologous to the exonuclease motifs in *E. coli* DNA polymerase I and other DNA polymerases (Blanco *et al.*, 1991; Derbyshire *et al.*, 1991). The UL30 subunit also possesses a RNase H activity (Crute and Lehman, 1989). This activity was confirmed by demonstrating that a recombinantly produced UL30 and a N-terminal proteolytic fragment of UL30 possessed the RNAse H activity (Marcy *et al.*, 1990; Weisshart *et al.*, 1994).

The UL42 subunit is a 488 amino acid, 51kDa phosphoprotein that possesses dsDNA binding activity (Boehmer and Lehman, 1997). The function of UL42 is to increase the processivity of the UL30 DNA polymerase (Gallo *et al.*, 1989; Gottlieb *et al.*, 1990; Hernandez and Lehman, 1990). UL42 acts as a DNA clamp which forms a ring that encircles the DNA duplex and interacts with the DNA polymerase to prevent dissociation of the polymerase from the primer-template, thereby ensuring high processivity. This clamp activity is functionally homologous to the β subunit of *E. coli* DNA polymerase III, eukaryotic proliferating cell nuclear antigen (PCNA) and

bacteriophage T4 gene 45 protein (Yao *et al.*, 1996). UL42 was shown to increase the affinity for a 3'-terminal primer by 10-fold (Gottlieb and Challberg, 1994)

The UL9 gene encodes for an 851 amino acid protein of 94kDa called the origin binding protein (OBP) (Boehmer and Lehman, 1997). OBP was identified by searching for a protein that could bind to sequences within oriS (Elias *et al.*, 1986). OBP is able to bind to two inverted repeats that flank the A+T rich region within oriS (Elias and Lehman, 1988). Amino acid sequence analysis of UL9 identified conserved ATP binding and DNA helicase motifs (Walker *et al.*, 1982; Gorbalenya *et al.*, 1989). UL9 functions as a homodimer and has a sequence specific DNA binding domain (Deb and Deb, 1991; Fierer and Challberg, 1992, 1995; Stabell and Olivo, 1993). The UL9 protein was shown to possess a DNA-stimulated nucleoside triphosphatase with a preference for ATP hydrolysis and DNA helicase activities (Bruckner *et al.*, 1991, Fierer and Challberg, 1992; Dobson and Lehman, 1993; Boehmer *et al.*, 1993). The DNA unwinding proceeds from 3'-5' (Fierer and Challberg, 1992; Boehmer *et al.*, 1993). The activities were stimulated by interaction with the UL29 (ICP8) single stranded DNA binding protein (Fierer and Challberg, 1992; Boehmer and Lehman, 1993; Boehemr *et al.*, 1993; Makhov *et al.*, 1996b). ICP8 appeared to stimulate the UL9 protein by preventing its dissociation from the DNA (Boehmer and Lehman, 1993b; Boehmer *et al.*, 1993). In the absence of ICP8, UL9 was not processive (Boehmer *et al.*, 1993). UL9 initiated structural changes in the DNA as was shown by DNAse I sensitivity in the A+T region of oriS when UL9 was bound (Elias *et al.*, 1990, Makhov *et al.*, 1996a). Electron microscopic analysis of UL9 bound to oriS demonstrated that stem-loop structures were extruded (Makhov *et al.*, 1996b). The ability of UL9 to recognize elements in the origins of replication and act as a DNA helicase suggests that it functions as replication initiator protein allowing entry of the DNA replication machinery into viral DNA.

The single stranded DNA binding protein, ICP8, is a product of the UL29 gene and consists of 1196 amino acids with a molecular weight of 128 kDa (Boehmer and Lehman, 1997). ICP8 binds to single-stranded DNA with at least a 5 fold greater affinity than double-stranded DNA (Ruyechan, 1983; Lee and Knipe, 1985). ICP8 has strand displacement activity as demonstrated by its ability to reduce the melting temperature of synthetic polynucleotides such as poly(dA)-poly(dT) and unwind short regions of duplex DNA (Bruce and Pearson, 1983; Boehmer and Lehman, 1993a). ICP8 can also catalyze the renaturation of complementary single strands of DNA (Dutch and Lehman, 1993). ICP8 can also transfer a DNA strand from a linear duplex to a complementary single-stranded DNA circle (Bortner *et al.*, 1993). The abilities of ICP8 to strand displace and reanneal complementary single strands are likely to be responsible for the ability for strand transfer (Bortner *et al.*, 1993). These activities may account for the high levels of homologous recombination observed in HSV-1 infected cells. ICP8 is also involved in important protein-protein interactions that may play a key role in assembly of the DNA replication complex such as the interaction with UL9 as described above.

The UL5, UL8 , and UL52 proteins are the three subunits of the DNA helicase-primase (Boehmer and Lehman, 1997). The holoenzyme consists of a 1:1:1 association of the 3 subunit proteins (Crute and Lehman, 1991). The helicase-primase was identified by its ability to unwind short oligonucleotides annealed to single-stranded M13 DNA in a 5'-3' direction (Crute *et al.*, 1988). The holoenzyme also has a DNA dependent ATPase activity that can also hydrolyze GTP (Crute *et al.*, 1988). The products of primase activity are oligoribonucleotides of 6-13 bp in length (Crute and Lehman, 1991; Sherman *et al.*, 1992; Tenney *et al.*, 1994). Using single-stranded ϕX174 DNA as a template, a preferred sequence for primase activity was identified and mapped to 3' AGCCCTCCCA (Tenney *et al.*, 1995). A 10-mer oligoribonucleotide

was produced starting at the underlined C (Tenney *et al.*, 1995). Amino acid sequence analysis of the UL5 protein led to the identification of conserved ATP-binding and DNA helicase motifs (Walker *et al.*, 1982; Gorbalenya *et al.*, 1989). Purified UL5 protein alone exhibited low levels of DNA-dependent ATPase activity (Sherman et al., 1992). A divalent metal binding motif in the UL52 protein was identified. This motif was conserved in DNA polymerases and primases (Klinedinst and Challberg, 1994; Dracheva *et al.*, 1995). Site-directed mutagenesis of this motif inactivated the primase activity of the holoenzyme and abolished origin-specific DNA replication (Klinedinst and Challberg, 1994; Dracheva *et al.*, 1995). The UL8 protein lacks detectable enzymatic or DNA binding activities (Dodson and Lehman, 1991; Parry *et al.*, 1993). However, UL8 protein was shown to stimulate primer synthesis by 3-fold but has no effect on the K_m for DNA or the rate of DNA-dependent ATPase and helicase activities of the UL5/UL52 core subunits (Sherman *et al.*, 1994; Tenney *et al.*, 1994, 1995; Gac *et al.*, 1996).

In addition to the 7 proteins identified as essential for DNA replications, there are several enzymes encoded by HSV-1 that are not required for replication in cultured cells but serve some purpose in DNA replication such as nucleic acid metabolism. These enzymes include uracil N-glycosylase, deoxyuridine triphosphatase, thymidine kinase, ribonucleotide reductase and alkaline nuclease (Boehmer and Lehman, 1997).

The HSV-1 uracil N-glycosylase is a 36 kDa protein encoded by the UL2 gene. The deamination of cytosine in DNA to uracil is a mutagenic event that results in the conversion of a G-C base pair to an A-T base pair. The uracil-N-glycosylase repairs this lesion by cleavage of the N-glycosidic bond linking uracil to the deoxyribose in the DNA backbone. This produces an abasic site that is repaired by AP endonuclease, DNA polymerase, and DNA ligase (Caradonna *et al.*, 1987; Vassyleyev and Morikawa, 1996; Krokan *et al.*, 2000).

The deoxyuridine triphosphatase (dUTPase) is a 39 kDa protein encoded by the UL50 gene (Williams, 1984; Caradonna and Adamkiewicz, 1984; Bjornberg *et al.*, 1993). The dUTPase catalyzes the hydrolysis of dUTP to dUMP and inorganic pyrophosphatase thus providing a mechanism to prevent incorporation of uracil into DNA. Also, the dUMP is a precursor of dTTP (Vassylyev and Morikawa, 1996).

The thymidine kinase (TK) is a homodimer of two copies of a 40 kDa protein encoded by the UL23 gene. TK catalyzes the phosphorylation of pyrimidine and purine deoxynucleosides as well as a wide variety of ribo- and deoxynucleoside analogs (Chen and Prusoff, 1978; Chen *et al.*, 1979). This broad substrate specificity is the basis for the therapeutic action of the acyclic guanosine analogs acyclovir and gancyclovir. Acyclovir is first phosphorylated by TK to generate the monophosphate. Cellular kinases further phosphorylate the monophosphate into the di- and triphosphate forms. Acyclovir triphosphate is a substrate for viral DNA polymerase and its incorporation results in chain termination and, consequently, inhibition of DNA replication (Reardon and Spector, 1989, Reardon, 1989).

The ribonucleotide reductase consists of two subunits of 124 kDa and 38 kDa encoded by UL39 and UL40, respectively. The ribonucleotide reductase functions to reduce ribonucleotides to deoxyribonucleotides creating a pool of substrates for DNA synthesis.

The UL12 gene encodes for a 85 kDa protein called alkaline nuclease (AN) (Costa *et al.*, 1983; Draper *et al.*, 1986, McGeoch *et al.*, 1988; Banks *et al.*, 1983). AN posseses both exonuclease and endonuclease activities that are optimally active at an alkaline pH (Hoffmann and Cheng, 1978, 1979; Strobel-Fidler and Francke, 1980; Banks *et al.*, 1983). AN plays a role in the egress of capsids from the nucleus of an infected cell (Shao *et al.*, 1992). AN will be discussed in more detail in section 1.4.

In addition to virally encoded enzymes, HSV replication also utilizes some cellular enzymes such as DNA ligase and DNA topoisomerase. The viral genome does not encode these two enzymes. Viral proteins alone cannot replicate viral DNA *in vitro* to any significant extent (Boehmer and Lehman, 1997).

1.3.6 Late Genes, Formation of Virions, and Egress.

HSV late (L) genes encode structural proteins and proteins involved in the assembly and egress of virions from an infected cell. The L genes can be further divided into two groups called early late (EL or γ_1) and delayed late (DL or γ_2) which differ in their timing and dependence on viral DNA synthesis for expression. DL gene expression is strictly dependent on the onset of viral DNA synthesis while EL genes are expressed prior to DNA replication, but their expression is enhanced by the onset of DNA synthesis (Roizman and Sears, 1996).

The assembly of capsids takes place within the nucleus of infected cells. Capsids are approximately 120nm in diameter and composed of 162 capsomers (Schrag *et al.*, 1989; Booy *et al.*, 1991; Trus *et al.*, 1992; Newcomb *et al.*, 1993; Zhou *et al.*, 1994). The capsid shell is composed of four predominant protein components, a major capsid protein (VP5), and three less abundant proteins (VP19C, VP23, and VP26) (Gibson and Roizman, 1972; Newcomb *et al.*, 1993). VP5, the product of the UL19 gene, is the major structural subunit of the capsomers, both hexons and pentons (Schrag *et al.*, 1989; Newcomb *et al.* , 1993). The 150 hexons in the HSV capsids are composed of 6 copies of VP5 while the 12 pentons are composed of 5 copies of VP5. The hexons are located at the edges and faces of the capsid shell, while the pentons are located at the vertices. The VP19C and VP23 proteins, products of the UL38 and UL18 genes, respectively, form the triplexes which are usually composed on one copy of VP19C and two copies of VP23 (Newcomb *et al.*, 1993). The triplexes form

intercapsomeric fibers that connect the capsomers together to stabilize the structure (Trus *et al.*, 1996). The VP26 protein (UL35 gene product) is located at the distal tips of the hexons, with one VP26 molecule bound to each hexon-associated VP5, and acts to stabilize the capsid (Booy *et al.*, 1994, Zhou *et al.*, 1995, Trus *et al.*, 1995).

Infected cells produce 3 distinct types of capsids called A, B, and C (Gibson and Roizman, 1972). They are distinguishable morphologically in electron microscopy and can be separated by sucrose density gradient ultracentrifugation. All three capsid types have the same basic shell structure as described above but differ in the composition of material inside the capsid cavity. C-capsids contain viral DNA and can mature into infectious virions (Perdue *et al.*, 1976). A and B capsids lack DNA. The B capsid cavity is filled primarily with VP22a, a cleaved form of the scaffolding protein (Newcomb and Brown, 1991), while A capsids lack significant amounts of protein. B capsids are believed to be procapsids that are able to package DNA and subsequently mature into C-capsids (Perdue *et al.*, 1976) while A capsids are believed to be an abortive form that results from failed attempts to package DNA (Sherman and Bachenheimer, 1988).

As mentioned above, the B capsids contain a large amount of the scaffolding protein VP22a, a product from the UL26.5 gene. In addition, B capsids contain VP24 and VP21, products from the UL26 gene (Gibson and Roizman, 1972; Newcomb *et al.*, 1993). The UL26 gene product is a protease that cleaves itself to generate VP24 and VP21 (Preston *et al.*, 1983; Dilanni *et al.*, 1993; Weinheimer *et al.*, 1993; Gao *et al.*, 1994; Liu and Roizman, 1993, 1995). Also, VP22a is obtained by the UL26 protease cleavage of the UL26.5 gene product (Liu and Roizman, 1995). During capsid assembly, the scaffolding proteins (VP22a, VP24, and VP21) form a core (scaffold) around which the capsid shell self-assembles. The scaffold proteins can interact directly with VP5 through their C-terminal 14 amino acids (Hong *et al.*, 1996).

This structure is unstable until the triplexes and UL26 protein are recruited and assembled into the structure. The scaffold proteins VP22a and VP21 are then cleaved by the UL26 protease at their C-termini and are removed from the capsid before or during entry of viral DNA. These steps have been confirmed by *in vitro* assemby of capsids using baculovirus infected cells or purified proteins (Thomsen *et al.*, 1994; Newcomb *et al.*, 1994; Tatman *et al.*, 1994).

Packaging of viral DNA involves cleaving of concatemers generated by DNA replication into unit-length molecules and insertion of the unit-length DNA into preformed capsids. At least seven gene products are involved in the encapsidation process. The products of the UL6, UL15, UL17, UL25, UL28, UL32 and UL33 genes were identified. Viral strains with mutations in any of these genes replicated DNA to near wild-type levels, however, concatemeric DNA and empty capsids accumulated, indicating that cleavage and packaging into preformed B capsids was defective (Tenglesen *et al.*, 1993; Patel *et al.*, 1996; Yu *et al.*, 1997; Lamberti and Weller, 1998; McNab *et al.*, 1998; Salmon *et al.*, 1998; Reynolds *et al.*, 2000). The UL32 and UL17 proteins might have a role in an early phase of the encapsidation process. These proteins appear to affect the localization of capsids and capsid proteins to viral replicative sites (Lamberti and Weller, 1998; Taus *et al.*, 1998). The UL15 and UL28 proteins appear to have a role in cleavage of progeny DNA. UL15 is homologous to bacteriophage T4 gp17, one of the terminase genes (Yu and Weller, 1998a). The UL15 gene contains two exons and encodes several different-sized proteins (Salmon and Baines, 1988; Yu *et al.*, 1997). UL15 and UL28 proteins were found to interact since some proteins of UL15 failed to localize to B capsids when the UL28 protein was not expressed (Salmon and Baines, 1998; Yu and Weller, 1998b). UL28 protein can mediate the nuclear import of UL15 proteins (Koslowski *et al.*, 1999). The UL6 protein is found in B and C capsids and, like the UL28 protein, the

UL6 protein influenced the cellular localization of UL15 proteins (Salmon and Baines, 1998; Yu and Weller, 1998b).

The UL25 protein does not appear to play a role in cleavage. HSV-1 mutants defective in UL25 cleaved DNA but did not package the DNA (McNab *et al.*, 1998). Also, there was an increase in A capsids suggesting that UL25 may play a role in stabilizing the DNA filled capsids late in the encapsidation process (McNab *et al.*, 1998). Very little is known about the UL33 protein except that it is essential for DNA packaging (Al-Kobaisi *et al.*, 1991). UL33 is a 19 KDa protein that localizes to the cytoplasm and replication compartments in the nucleus, but was not detected in purified virions or capsids (Reynolds *et al.*, 2000) . In addition to the above 7 gene products, the UL12 alkaline nuclease plays an important but not essential role in the packaging process (Weller *et al.*, 1990; Shao *et al.*, 1993). The alkaline nuclease was proposed to function in resolving the branched DNA formed during replication (Shao *et al.*, 1993; Martinez *et al.*, 1996a, Severini *et al*, 1996). Alkaline nuclease will be discussed in greater detail in section 1.4.

The cleavage of concatemers occurs at sites within the "a" sequences found at both ends of the genome and as well as the L/S junction (Stow *et al.*, 1983; Varmuza and Smiley, 1985; Deiss *et al.*, 1986; Deiss and Frenkel, 1986; fig 1.2). The a sequence contains both unique (U) and directly repeated (DR) elements and can be represented as DR1-U_b-$DR2_n$-$DR4_m$-U_c-DR1 (Roizman and Sears, 1996). In different HSV-1 strains, or even in the same strain, the size of the a sequence varies from 250 to 500bp which reflects the variability of the copies of DR elements. Within the unique U_b an U_c elements are two blocks of sequences, called pac1 and pac2, respectively, which are essential for cleavage and packaging of newly replicated DNA (Stow *et al.*, 1983; Varmuza and Smiley, 1985; Deiss *et al.*, 1986). Viral DNA is spooled into a preformed capsid until a second a sequence of the same polarity is

encountered. Pac1 defines a cleavage site at a distance of 40-44 nucleotides in a DR1 element at the S terminus, whereas pac2 defines a cleavage site at a distance of 30-35 nucleotides in a DR1 element at the L terminus (Varmuza and Smiley, 1985; Deiss *et al.*, 1986). The cleavage process appears to be accompanied by the amplification of an "a" sequence. This was shown when molecules that carry junctions of the sequence"xay" were cleaved to generate "xa" and "ay" termini (Deiss and Frenkel, 1986).

The final step in the life cycle is egress of virions. It is agreed that mature HSV capsids bud through modified patches of the inner nuclear membrane, delivering enveloped virions into the space between the inner and outer nuclear membrane (Schwartz and Roizman, 1969; Torrisi *et al.*, 1992). The subsequent steps have been described by two opposing models. In one model, the enveloped virion transits the endoplasmic reticulum and Golgi network through the secretory apparatus (Johnson and Spear, 1982). According to this model, the envelope is derived from the inner nuclear membrane and the tegument components and envelope glycoproteins are incorporated into virions as they bud out of the nucleus. The alternative model proposes that the initial virion envelope fuses with the outer nuclear membrane delivering a naked capsid into the cytoplasm (Komuro *et al.*, 1989; Whealy *et al.*, 1991; van Gendersen *et al.*, 1994; Browne *et al.*, 1996; Whitley *et al.*, 1999). The capsid acquires its final envelope and tegument as it buds into cytoplasmic vesicles. Regardless of the mechanism, several proteins have been identified to play a role in egress such as UL11, UL20, and UL53 (gK) (Baines *et al.*, 1991; Baines and Roizman, 1992; Hutchinson and Johnson, 1995; Jayachandra *et al.*, 1997) . The precise functions of these proteins is not currently known but mutants in these genes show defects in envelopment.

1.4 HSV-1 Alkaline Nuclease

Infection of mammalian cells by herpes simplex viruses results in the induction of a novel nuclease (Keir and Gold, 1963). The nuclease is distinguished from host enzymes due to its optimal activity at high pH and was named alkaline nuclease (AN) (Keir and Gold, 1963; Morrison and Keir, 1968). Confirmation that AN is encoded by a HSV gene was supported by the isolation of a temperature sensitive mutant of HSV-2 that does not produce AN at a nonpermissive temperature (Francke *et al.*, 1978). Also, microinjection of plasmids containing HSV DNA into *Xenopus laevis* oocytes resulted in the production of AN (Preston and Cordingley, 1982). AN has been further characterized as having both an exonuclease and endonuclease activities, an absolute requirement for divalent cation (either Mg^{2+} or Mn^{2+}) and a reducing agent for activity. AN activity was inhibited by NaCl or KCl (Morrison and Keir, 1968; Hoffmann and Cheng, 1978, 1979; Strobel-Fidler and Francke, 1980; Banks *et al.*, 1983). The exonuclease activity can digest single and double-stranded linear DNA producing 5' monophosphonucleotides in the 5' to 3' direction (Hoffmann and Cheng, 1978; Strobel-Fidler and Francke, 1980; Hoffmann, 1981; Banks *et al.*, 1983; Knopf and Weisshart, 1990). When Mg^{2+} was used in the assays, both endonuclease and exonuclease activities were detected, but in the presence of Mn^{2+}, endonuclease activity was predominant (Hoffmann and Cheng, 1979). Under these conditions, AN was found to preferentially digest supercoiled DNA over non-supercoiled DNA. In all the above studies, no sequence specificity was detected. AN is a phosphoprotein, phosphorylated by the HSV US3 protein kinase (Daikoku *et al.*, 1995), and has an apparent molecular weight of 85kDa (Banks *et al.*, 1983, 1985). AN was found in the nucleus of an infected cell, but does not localize to distinct replication compartments

with other replication proteins. Instead, it remains diffusely distributed throughout the nucleus (Puvion-Dutilleul and Pichard, 1986; Randall and Dinwoodie, 1986).

The AN gene has been mapped to 0.168 to 0.175 map units on the genome by marker rescue of a temperature sensitive mutant (Moss *et al.*, 1979), transient expression of fragment of viral DNA in *Xenopus laevis* oocytes (Preston and Cordingley, 1982), analysis of deletion mutants (Wathen and Hay, 1984) and HSV-1 X HSV-2 recombinants (Banks *et al.*, 1985). A 2.3 kb mRNA was identified that mapped to this region and produced a 82kDa polypeptide that was immunoprecipitated by a monoclonal antibody against the AN of HSV-2 (Costa *et al.*, 1983). This region encodes for a family of unspliced 3' coterminal mRNAs of 2.3kb, 1.9kb, and 0.9kb. The DNA sequence of this region has been analyzed and an open reading frame encoding for a product of 626 amino acids was identified within the 2.3kb mRNA and corresponds to UL12 (Draper *et al.*, 1986; McGeoch *et al.*, 1986, 1988). The 1.9kb mRNA encodes for a N-terminally truncated 60kDa protein that shares the C-terminus with AN and was designated UL12.5 (Martinez *et al.*, 1996b). This protein has both endonuclease and exonuclease activities with the same pH optimum, ionic strength, and divalent cation requirements as AN (Bronstein *et al.*, 1997).

The role of alkaline nuclease in the viral life cycle was studied initially with a temperature sensitive (ts) mutant of HSV-2 AN (Timbury, 1971). The mutant was found to have a mutation that causes irreversible inactivation of AN activity at a nonpermissive temperature. Analysis of viral DNA synthesis in ts mutant infected cells at the nonpermissive temperature (38.5^{o}C) showed that viral DNA was synthesized in the absence of active AN, but less efficiently than in the presence of active AN (Francke *et al.*, 1978; Moss *et al.*, 1979; Francke and Garrett, 1982). These results indicated that AN was not essential for replication of viral DNA but may enhance its synthesis. However, a problem with these mutants is their leakiness. A small amount

of AN activity produced at the nonpermissive temperature might be sufficient for viral growth (Moss *et al.*, 1979; Francke and Garrett, 1982). At higher temperatures (39.2°C), Moss (1986) reported that the level of AN activity and viral DNA synthesis were considerably reduced. Marker rescue of the ts lesion in AN restored wild type levels of viral DNA synthesis and growth which suggested that AN was essential for virus replication (Moss, 1986).

To resolve the different interpretations of the necessity of AN in viral replication and circumvent the problems with leaky ts mutants, a null mutant of HSV-1 AN was created (Weller *et al.*, 1990). The mutant, AN-1, had an insertional mutagen consisting of the lacZ gene of *Escherichia coli* under the control of the regulatory elements of the large subunit of ribonucleotide reductase replacing 917 bp from the N-terminal half of UL12. A stop codon at the end of lacZ ensured that the remaining portion of AN was not expressed. Viral DNA and late protein synthesis were near wild-type levels. This indicated that AN was not essential for HSV DNA synthesis. This observation was in agreement with the findings that UL12 was not one of the seven genes identified as being necessary for viral DNA synthesis (UL5, UL8, UL9, UL29, UL30, UL42, and UL52) (Wu *et al.*, 1988). The AN-1 mutant formed tiny plaques and the production of infectious virus in nonpermissive cells was severely compromised (100- to 1000-fold less than wild-type). This suggested that AN-1 may play a role in the processing or packaging of viral DNA to produce infectious virions.

Several steps in genome maturation and encapsidation of viral DNA were examined in cells infected with wild-type HSV-1 and AN-1 to try and determine what role AN may play in these processes (Shao *et al.*, 1993; Martinez *et al.*, 1996a). Cleavage of viral genomes was examined by hybridization to probes for terminal fragments. Cleavage in the AN-1 mutant was as efficient as cleavage in the wild-type virus. To examine encapsidation, the amount of DNase I resistant DNA was compared

and it was found that a significant amount of AN-1 DNA remained after DNase I treatment which indicated that packaging had occurred. Another way encapsidation was studied compared the amount of staphylococcal nuclease (SN) resistant viral DNA in cytoplasmic and nuclear cell fractions. In wild-type infected cells, SN resistant and sensitive DNA was found in nuclear fractions and only SN resistant DNA was found in the cytoplasm indicating that all the cytoplasmic DNA was encapsidated. However, very little SN resistant DNA was found in the cytoplasm of AN-1 infected cells indicating that encapsidated DNA failed to egress efficiently into the cytoplasm. This was also seen in electron microscopy of AN-1 infected cells, where capsids were seen in the nucleus but very few capsids were seen in the cytoplasm. Very few mature virions were observed at the cell surface. The capsid forms of nuclear and cytoplasmic fractions were analyzed after separation on sucrose gradients. In wild-type infected cells, the major capsid species in the nucleus was B capsids, with a minority of A capsids. In AN-1 infected cells, however, A and B capsids were present in approximately the same amount in the nucleus. In the cytoplasm of wild-type infected cells, a large amount of B and C capsids and a minor amount of A capsids were observed but no capsids are seen in the cytoplasm of AN-1 infected cells. It was believed that one or more steps in the production of capsids were defective in AN-1 infected cells. These capsids may be aberrant or unstable and disgorge the viral DNA that was being packaged, leading to the production of A capsids (Shao *et al.*, 1993)

A possible reason for the formation of aberrant capsids is that the DNA is altered in either size or structure. Martinez *et al.* (1996a) examined the structure of replicating DNA in AN-1 infected cells. The structure of wild-type replicating viral DNA has been shown be be a large complex and branched mass (Shlomai *et al.*, 1976; Friedman *et al.*, 1977; Severini *et al.*, 1994, 1996; Zhang *et al.*, 1994). The majority of replicating DNA is unable to migrate from the well of a gel during pulsed-field gel

electrophoresis. Digestion of the DNA with a restriction enzyme that cuts once per genome releases some monomer length viral DNA, but the majority of the DNA still remains in the well (Severini *et al.*, 1994; Zhang *et al.*, 1994). In AN-1 infected cells, digestion with single site per genome restriction enzymes releases no discrete fragments, suggesting that the replicative intermediates in AN-1 are even more complex than in wild-type (Martinez *et al.*, 1996a). This complex, branched replicating viral DNA poses a problem for packaging as a monomer genome must be released before or during packaging. A Holliday junction is the key intermediate in homologous recombination (Holliday, 1964). During homologous recombination, two linear duplexes become linked by a single crossover due to strand invasion. This junction is cleaved by an endonuclease, called a Holliday junction resolvase, for resolution of the recombination intermediate into two linear DNA duplexes. It was found that T7 endonuclease I could remove the branches (Severini *et al.*, 1996). T7 endonuclease I is a Holliday junction resolvase (deMassey *et al.*, 1987; Dickie *et al.*, 1987; Picksley *et al.*, 1990). It has been proposed that AN resolves the branches formed during replication serving as a Holliday resolvase (Shao *et al.*, 1993; Martinez *et al.*, 1996a; Severini *et al.*, 1996). It follows that the defect in AN-1 would result in the inability to remove these branches producing aberrantly packaged capsids which would be unable to egress from the nucleus.

In order to further define the role of AN, AN was expressed in a heterologous system. Recombinant expression has been accomplished in rabbit reticulocyte lysate (Henderson *et al.*, 1998), baculovirus (Kehm *et al.*, 1998a; Goldstein and Weller, 1998a), *Escherichia coli* (Bronstein and Weber, 1996), vaccinia virus and Semliki Forest virus (Kehm *et al.*, 1998b) expression systems. The AN produced in these expression systems had the same endo-exonuclease activity and biochemical characteristics as AN purified from HSV-1 infected cells. Mutagenesis of the UL12

gene and expression in a heterologous system has been used to identify domains or residues important for the function of AN. Searches for homology to other proteins was done. Within the herpesviruses, there are homologs of UL12 (Martinez *et al.*, 1996b; Goldstein and Weller, 1998a). The UL12 homologs from Epstein-Barr virus, human cytomegalovirus, pseudorabies virus, and bovine herpesvirus type 1 have been shown to have similar endo-exonuclease activities to the AN of HSV-1 (Baylis *et al.*, 1989, 1991; Stolzenberg and Ooka, 1990; Lin *et al.*, 1995; Schaeffer *et al.*, 1997; Hsiang *et al.*, 1996; Chung and Hsu, 1997). The human cytomegalovirus homolog, UL98, was able to complement the defect in a null mutant of AN (Gao *et al.*, 1998). Sequence alignments have identified 7 conserved regions or motifs among the UL12 homologs (Martinez *et al.*, 1996b; Goldstein and Weller, 1996a; fig 4.1). Deletion of 126 amino acids from the N-terminal end of AN resulted in a mutant that had the same activity as wild type AN (Henderson *et al.*, 1998). As well, the UL12.5 protein, a product from the 1.9kb co-terminal mRNA within the UL12 open reading frame, lacks 126 amino acids from the N-terminus and maintained full endonuclease and exonuclease activities similar to full length AN (Bronstein *et al.*, 1997). All of the 7 conserved motifs are downstream of the N-terminal 126 amino acids, indicating that these regions may be important for nuclease activity. However, the truncated AN mutant cannot fully complement AN-1 (Henderson *et al.*, 1998) and a mutant HSV-1 that produces only UL12.5 has the same growth defect as AN-1 indicating that it could not substitute for full length UL12 (Martinez *et al.*, 1996b). The N-terminal region is proline rich and since proline rich regions are motifs for protein-protein interactions, this region may be needed to form stable contact with other viral or cellular proteins (Henderson *et al.*, 1998).

Point mutations have been generated in UL12 (Henderson *et al.*, 1998; Goldstein and Weller, 1998a). The L150K mutant (substitution of leucine at amino

acid 150 for lysine) (Henderson *et al.*, 1998) was not found within the conserved motifs. However, this mutant lacked nuclease activity and was unable to complement AN-1. Two mutants were constructed within conserved motif II (amino acids 325 - 340) (Goldstein and Weller, 1998a). The G366A/S338A (substitution of glycine 366 and serine 338 with alanine) lacked both exonuclease and endonuclease activities and was unable to complement AN-1. The other mutant made, D340E (substitution of aspartic acid 340 with glutamic acid), resulted in a product that lacked exonuclease activity, but retained endonuclease activity. The D340E mutant also failed to complement AN-1. This led Goldstein and Weller (1998a) to conclude that the major role of AN *in vivo* was probably exonucleolytic activity. A test for resolvase activity was performed with wild type AN and AN mutants G366A/S338A and D340E (Goldstein and Weller, 1998b). There was no resolution or degradation of the synthetic Holliday substrates with D340E or G336A/S338A. However, wild type AN simply degraded the branched structures instead of resolving them into linear forms. This result suggested that AN was not a Holliday resolvase. It was speculated that the exonuclease was involved in secondary structure repair or that AN degrades 5' single-stranded DNA tails which could invade duplex DNA and, unlike 3' ends, cannot be used as primers for DNA synthesis (Goldstein and Weller, 1998b). Based on these results, the role of AN in the viral life cycle still remains unclear.

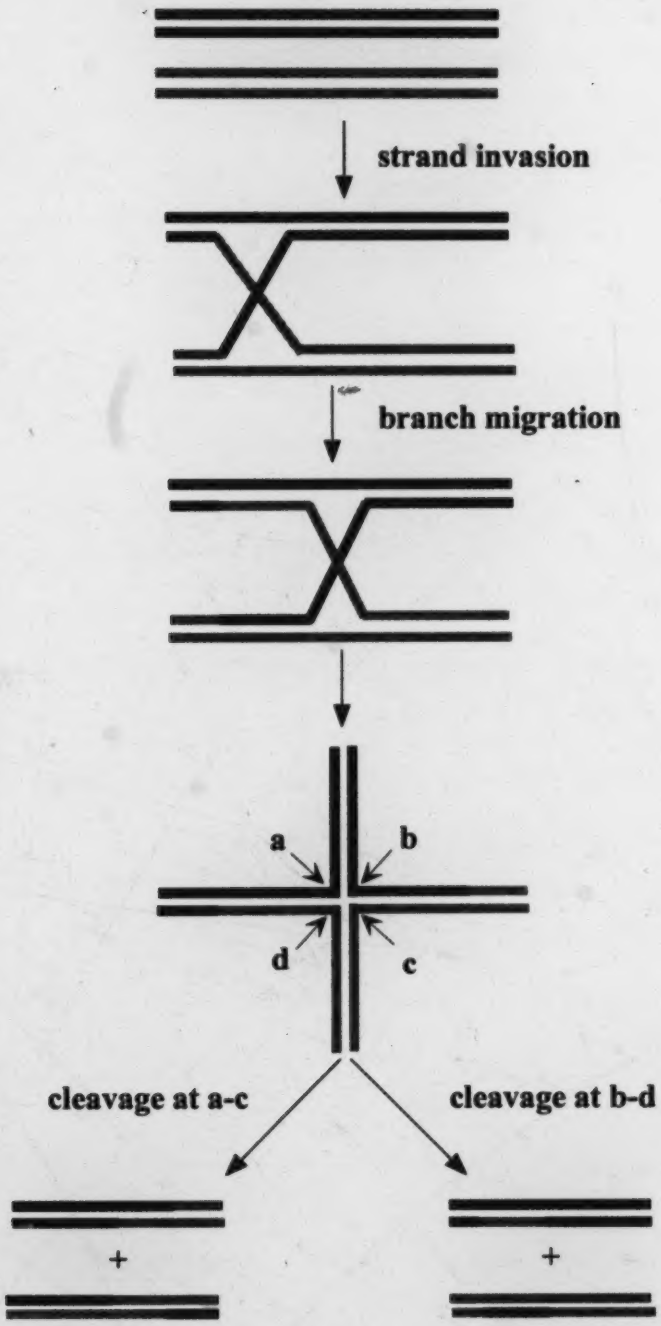
1.5 Holliday Junctions and Resolvase

Recombination between homologous DNA is one of the cellular processes responsible for the generation of genetic diversity. In addition, recombination provides a mechanism for the repair of DNA breaks or lesions caused by irradiation or harmful chemicals (reviewed by West, 1997). The process of homologous recombination has also been usurped to serve other purposes such as ensuring proper segregation of chromosomes during cell division and initiation of DNA replication in viruses, plasmids, bacteria, mitochondria, chloroplasts, and yeast (Viret *et al.*, 1991; Preiser *et al.*, 1996; Kogama, 1997; Mosig, 1987, 1998)

The process of homologous recombination involves interaction between two DNA segments that are identical, or nearly identical, to each other. Breakage of the DNA backbone followed by strand exchange events form a heteroduplex intermediate in which strands from each parental molecule become base paired with the other partner. This intermediate, in which two recombining molecules become linked by a single crossover, is the key intermediate in homologous recombination as originally proposed by Robin Holliday (1964). This structure is commonly referred to as a Holliday junction. As shown in figure 1.4, branch migration extends the length of the heteroduplex. This structure is equivalent to the four stranded structure seen in figure 1.4, and called a cruciform. Recombination is completed by cleavage of the cruciform by enzymes called Holliday resolvases to generate two linear duplex recombinant products. Various elaborations of this basic model have been proposed (Meselson and Radding, 1975: Orr-Weaver *et al.*, 1981), but a four stranded Holliday junction is a common feature of all of the models.

1.5.1 Formation of Holliday Junctions.

Figure 1.4 Steps in homologous recombination. Homologous recombination is initiated by invasion of homologous DNA with single-stranded DNA and exchange between two duplexes creating a Holliday junction. The heteroduplex is extended through branch migration by helicase. This structure is equivalent to the four stranded cruciform structure. Resolution of the homologous recombination structure involves cleavage by a Holliday resolvase at either a-c or b-d to generate two unconnected DNA duplexes.


strand invasion
branch migration
a
b
d
c
cleavage at a-c
cleavage at b-d
+
+

The formation of a Holliday junction is best understood in *E. coli* and will be briefly described. Recombination events are initiated by single-stranded DNA (ssDNA) or partially ssDNA. The ssDNA is produced by the RecBCD enzyme. RecBCD, formerly known as exonuclease V, is a heterotrimer that possesses DNA helicase, double-stranded and single-stranded DNA exonuclease and single stranded endonuclease activities (Smith, 1990). Stimulation of recombination resulted from RecBCD-specific nicking event five nucleotides to the 3' side of the Chi sequence (5'-GCTGGTGG-3') (Dixon and Kowalczykowski, 1991). RecBCD was believed to bind the ends of linear duplex DNA and begin unwinding through its helicase activity (Roman *et al.*, 1992). As it unwound, RecBCD cleaved primarily the 3' strand of the DNA endonucleolytically. When RecBCD encountered a Chi site in the correct orientation, it paused and a nick was introduced (Tayor *et al.*, 1985). The result of the interaction of RecBCD was attenuation of the 3'-strand specific nuclease activity (Taylor and Smith, 1992; Dixon and Kowalczykowski, 1993). The attenuation was believed to be due to the inactivation or loss of the RecD subunit (Dixon *et al.*, 1994). The enzyme continued to track along the DNA with continuous unwinding. The result was the production of an intact ssDNA tail with a 3' end. This ssDNA was bound by the RecA protein which formed a helical nucleoprotein filament capable of homologous pairing and strand invasion resulting in formation of recombination intermediates containing Holliday junctions (Biano *et al.*, 1998).

Branch migration to extend the length of the heteroduplex created was mediated by the actions of RecG, RuvA and RuvB proteins. RuvA and RuvB worked together in a complex (Shiba *et al.*, 1991). The RuvA protein bound both single- and double-stranded DNA but bound with the greatest affinity to Holliday junctions (Shiba *et al.*, 1991, Iwasaki *et al.*, 1992; Parsons *et al.*, 1992). RuvA targeted RuvB to the junction (Parsons and West, 1993). RuvB has DNA-dependent ATPase and DNA helicase

activities (Gorbalenya and Koonin, 1993; Marrione and Cox, 1995). RuvB bound weakly to DNA and promoted branch migration (Tsaneva *et al.*, 1992. Muller *et al.*, 1993b), however, formation of a RuvAB complex enhanced helicase activity (Muller *et al.*, 1993a). RuvAB was able to promote ATP-dependent branch migration in synthetic Holliday junctions (Iwasaki *et al.*, 1992; Parsons *et al.*, 1992; Tsaneva *et al.*, 1992; Muller *et al.*, 1993a; Parsons and West, 1993). The recG gene encodes a protein that bound to Holliday junctions and promoted ATP-dependent branch migration (Lloyd and Sharples, 1993; Whitby *et al.*, 1994). RecG has activities that partially overlap the Ruv proteins as shown by the observation that recombination was more severely depressed in ruv recG double mutants and that these mutant strains were more sensitive to ultraviolet radiation than were either ruv or recG single mutants (Lloyd, 1991).

As previously mentioned above, the process of recombination is utilized by some bacteriophages as a mechanism for initiating DNA replication. This also requires the formation of Holliday junctions and is best studied in the bacteriophage T4 (Mosig, 1987, 1998). The bacteriophage T4 has a double stranded linear genome of approximately 171 kb. Replication resulted in the formation of head-to-tail concatemeric DNA that were cut to unit length by a terminase before packaging into preformed heads (Mosig, 1987, 1998). For T4 , and other related phages (such as T7), homologous recombination is essential because these viruses have to use intermediates of recombination to initiate most of their replication forks even under favorable conditions for growth. The first round of replication is initiated at specific origin sequences and requires RNA primers (Luder and Mosig, 1982). When the first growing point reaches one end of the genome, the 3' end remained single-stranded. This 3' end invaded a homologous DNA segment . This invading 3' end served as a primer for new replication forks (Luder and Mosig, 1982). When this replication fork reaches the end of the template, one of the forks will have a single-stranded end and the

process can be repeated by another invasion. Ultimately, this recombination-initiated replication generated a branched DNA network (Luder and Mosig, 1982). These branches are analogous to Holliday junctions. The T4 UvsX protein is a RecA homolog that bind to ssDNA and forms filaments similar to RecA protein in an ATP-dependent process (Hashimoto and Yonesaki, 1991; Biano *et al.*, 1998). These filaments facilitated the search for homology and invasion of ssDNA into dsDNA. This activity was stimulated by the UvsY protein (Morrical and Alberts, 1990; Hashimoto and Yonesaki, 1991). Branch migration is catalyzed by the helicase gp41 (Salinas and Kodadek, 1995), which was loaded onto DNA by gp59 (Mosig, 1998) and the UvsW helicase (Carles-Kinch *et al.*, 1997). The T4 uvsW gene was able to complement *E. coli* recG mutants (Carles-Kinch *et al.*, 1997).

1.5.2 Resolution of Holliday Junctions

Recombination is completed by resolving Holliday junctions to release mature recombinant products. Resolution occurs by matched cleavages across the axis of the branch point in a Holliday junction, as illustrated in figure 1.4, by cutting DNA strands a-c or b-d. Enzymes called resolvases are required for these specific cleavages (White *et al.*, 1997). Junction resolving enzymes are ubiquitous nucleases found in sources ranging from bacteriophages (Mizuuchi *et al.*, 1982; Lilley and Kemper, 1984; Kemper *et al.*, 1984; deMassey *et al.*, 1987, Dickie *et al.*, 1987), to bacteria (Connolly *et al.*, 1991; Dunderdale *et al.*, 1991, 1994; Iwasaki *et al.*, 1991; Sharples and Lloyd, 1991), yeast (West and Korner, 1985, Symington and Kolodner, 1985; Evans and Kolodner, 1987; Parsons and West, 1988; Jensch *et al.*, 1989; Kleff *et al.*, 1992; Kupfer and Kemper, 1996; Oram *et al.*, 1998; Whitby and Dixon, 1997, 1998) , mammalian cells (Jeyaseelan and Shanmugan, 1988; Waldman and Liskay, 1988, Elborough and West; 1990; Couture and Chow, 1992; Hyde *et al.*, 1994), and eukaryotic viruses (Stuart *et*

al., 1992; Garcia *et al.*, 2000). In general, the structure, especially the four-way junction, is much more important than the base sequence (White *et al.*, 1997).

The first nuclease to be identified that was selective for branched DNA was encoded by phage T4 gene 49 (Kemper and Janz, 1976). Mutants in gene 49 were found to produce a multiply-branched form of DNA which could not be packaged into phage heads (abortive infection) (Kemper and Janz, 1976; Kemper and Brown, 1976). The product of gene 49 is a 157 amino acid, 18kDa protein called endonuclease VII (Kemper and Garabett, 1981). Thus endonuclease VII was required to cleave branched DNA into unit-length phage molecules before packaging. This nuclease resolved DNA recombination intermediates in plasmids and cruciform DNA structures (Mizuuch *et al.*, 1982; Lilley and Kemper, 1984; Kemper *et al.*, 1984). In addition, T4 endonuclease VII cleaved a variety of branched DNA structures including Y junctions, heteroduplex loops, extended single strand termini, and sequence induced bends in duplex DNA (Jensch and Kemper, 1986, Kleff and Kemper, 1988; Meuller *et al.*, 1990). This broad specificity was believed to be consistent with its role as a general debranching and repair enzyme that prepared phage DNA for packaging. Although the interaction with junctions appeared to be structure specific, endonuclease VII showed a slight sequence specificity and would generally introduce nicks to the 3' side of pyrimidine residues located between two to three nucleotides from the junction point (Pottmeyer and Kemper, 1992).

Another well characterized resolvase is the RuvC protein from *E. coli*. The RuvC protein contains 173 amino acids and has a molecular weight of approximately 18.7 kDa and forms a dimer (Dunderdale *et al.*, 1991; Sharples and Lloyd, 1991). There was no sequence homology with T4 endonuclease VII. The RuvC protein has been purified and resolved intermediates made by RecA, synthetic Holliday junctions produced from oligonucleotides, and cruciform structures extruded from plasmids

(Connolly *et al.*, 1991; Dunderdale *et al.*, 1991, 1994; Iwasaki *et al.*, 1991). Like endonuclease VII, RuvC had a slight sequence specificity (Bennett *et al.*, 1993; Shah *et al.*, 1994). Cleavage occured preferentially at the sequence 5'-A/T TT G/C-3', where scission took place after the two central T residues. Unlike endonuclease VII, RuvC failed to cleave Y-structures, mismatches, and heteroduplex loops (Benson and West, 1994). Also, no nuclease activity was observed with single-stranded or linear duplex DNA (Dunderdale *et al.*, 1994). The crystal structure of RuvC has been determined (Ariyoshi *et al.*,1994). The dimer of RuvC formed a large cleft that accomodated duplex DNA. At the bottom of the cleft were 4 acidic residues involved in coordination of a metal cation (Mg^{2+}) and were important for catalysis (Saito *et al.*, 1995). This was confirmed by mutagenesis of these residues (Sharples and Lloyd, 1993; Saito *et al.*, 1995). Also within this cleft are eight residues (lysine and arginine) whose side chains were thought to be involved in DNA binding (Saito *et al.*, 1995).

1.6 Rationale and Objectives

Studies on the structure of the replicative intermediates of HSV-1 DNA have revealed that the intermediates are in a much more complex form than simple head-to-tail concatemers produced by rolling circle. Replicating DNA is highly branched and evidence was provided that these branches consisted of X- and Y-branches (Shlomai *et al.*, 1976; Friedman *et al.*, 1977; Severini *et al.*, 1994, 1996). This is reminiscent of the replication of the bacteriophage T4 (Mosig, 1987, 1998, section 1.5). During replication, the replicating T4 genome became highly branched due to single-stranded 3' DNA end invasion of homologous sequences to initiate DNA replication. This DNA was shown to contain many X- and Y-branched molecules. These branches must be removed for successful packaging. The T4 gene 49 performed the debranching function as demonstrated by the observation that mutants in gene 49 accumulated multiply branched forms of DNA that could not be packaged. The product of gene 49 is T4 endonuclease VII, which has been shown to function as a Holliday resolvase. Since HSV-1 DNA also has branched DNA, in this thesis I wanted to determine whether a Holliday resolvase activity is present in HSV-1 infected cells. This activity has not previously been reported from HSV-1 infected cells. It was interesting that T7 endonuclease I, another Holliday resolvase, was able to remove these branches from replicating HSV-1 DNA (Severini *et al.*, 1996). In chapter 1, a nuclease found in HSV-1 infected cell lysates but not uninfected cells was partially purified and found to possess both endonuclease and exonuclease activities. DNA substrates that were analogous to Holliday junctions were used to determine if this HSV-1 induced nuclease can cleave these structures in a manner consistent with Holliday resolvase activity.

The idea that HSV-1 AN may be the Holliday resolvase had been suggested from the studies of a null mutant called AN-1 (Weller *et al.*, 1990). It was found that,

although viral DNA synthesis was nearly as efficient as wild-type virus, the production of virions was severely restricted because capsids were unable to egress from the nucleus of an infected cell (Weller et al., 1990; Shao *et al.*, 1993). A defect in packaging was also supported by the observation that a much higher proportion of abortive capsids were produced compared to wild-type infection (Shao *et al.*, 1993). In addition, replicative intermediates in AN-1 were found to be even more branched than wild-type intermediates (Martinez *et al.*, 1996a). As mentioned above, this was reminiscent of mutants in phage T4 gene 49. However, it was shown that HSV-1 AN was not a Holliday resolvase (Goldstein and Weller, 1998b). Since in chapter 2, the enzyme preparation used was a crude preparation, it was possible that other viral or cellular proteins were responsible for resolvase activity. In chapter 3, using purified AN, Holliday resolvase activity was evaluated.

In chapter 4, point mutations were made in the UL12 gene and these mutant nucleases were characterized. The mutations were targeted to acidic residues found in the seven conserved motifs identified in UL12 (Martinez *et al.*, 1996b; Goldstein and Weller, 1998a). The acidic residues were selected for site-specific mutagenesis because previous studies had shown that acidic residues were important for catalytic activity in metal-ion coordinating nucleases (Suck, 1992; Gerit, 1993). Previously, a mutant in one of the conserved residues was produced that lacked exonuclease activity, but retained endonuclease activity (Goldstein and Weller, 1998a). Holliday resolvase was not demonstrated for this mutant (Goldstein and Weller, 1998b). It was my objective to produce similar types of mutants and to determine if some of them could demonstrate Holliday resolvase activity without exonuclease activity.

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Chapter 2
Detection of Holliday Junction Resolving Activity In Herpes Simplex Virus Type 1 Infected Cells

2.1 Introduction

Replication of herpes simplex virus DNA in the nucleus of an infected cell is believed to proceed by a rolling circle mechanism in which a long string of head-to-tail concatemers is produced. These are subsequently cleaved into linear monomers before packaging into capsids (Boehmer and Lehman, 1997; Roizman and Sears, 1996). In contrast, studies of the structure of replicating viral DNA by pulsed-field gel electrophoresis indicated that the replicative intermediates have a complex, possibly branched, structure (Severini *et al.*, 1994; Zhang *et al.*, 1994). Using neutral two-dimensional gel electrophoresis and electron microscopy, direct evidence for branches has been obtained (Severini *et al.*, 1996). These branches consist of Y- and X-junctions. The branches pose a problem for replicating DNA as these branches must be removed prior to or during packaging because mature viral DNA is linear and unbranched. The mechanism of the formation of the branches is unknown, but the replication of the bacteriophage T4 may provide some clues about the mechanism of debranching (Mosig, 1987, 1998).

The bacteriophage T4 is a large DNA virus that replicates via a rolling circle mechanism that produces concatemers. During the late phase of replication, genomic ends invade homologous sequences on other genomes creating replication forks. This recombination during replication results in a large network of branched concatemers

which must be debranched prior to packaging. Debranching of the genomes is performed by the product of T4 gene 49 called endonuclease VII (Mosig, 1987, 1998). Mutants in gene 49 produce highly branched, very fast sedimenting DNA complexes which contain an excess of Y- and X-junctions (Kemper and Janz, 1976; Kemper and Brown, 1976; Flemming *et al.*, 1993). T4 endonuclease VII is known to act as a Holliday junction resolvase (Mizuuchi *et al.*, 1982; Kemper *et al.*, 1984) Holliday junctions are the key intermediates in homologous recombination (Holliday, 1964). These junctions are formed by the reciprocal exchange of single strands between two homologous DNA duplexes resulting in two duplexes linked by a single crossover (chapter 1, fig 1.4). Resolution requires the endonucleolytic cleavage of this junction by a resolvase to produce two unconnected duplexes. Bacteriophage T7 endonuclease I is an enzyme similar to T4 endonuclease VII (de Massey *et al.*, 1987; Dickie *et al.*, 1987b) and has been shown to be able to remove the branches in replicating HSV-1 DNA (Severini *et al.*, 1996).

A Holliday resolvase has not previously been described in HSV-1 infected cells but it has been suggested that HSV-1 alkaline nuclease (AN) provides an analogous function to T4 endonuclease VII in debranching (Weller *et al.*, 1990; Shao *et al.*, 1993; Martinez *et al.*, 1996; Severini *et al.*, 1996) . HSV-1 AN is the product of the UL12 open reading frame (Costa *et al.*, 1983; Draper *et al.*, 1986; McGeoch *et al.*, 1986, 1988). It is a relatively abundant phosphoprotein found in infected cells. AN is a 626 amino acid protein with an apparent molecular weight of 85 kDa and possesses both exonuclease and endonuclease activities that are optimal at a high pH (Morrison and Keir, 1968; Hoffmann and Cheng, 1978, 1979; Strobel-Fidler and Francke, 1980; Banks *et al.*, 1983). Studies with a null mutant, AN-1, indicated that AN plays a role in the processing and packaging of viral DNA (Weller *et al.*, 1990; Shao *et al.*, 1993). Although AN-1 synthesizes near wild type levels of both viral DNA and late proteins, it

is severely restricted in the production of mature virions. Viral DNA is efficiently packaged into capsids but these capsids are unable to egress from the nucleus of an infected cell. Furthermore, the replicative intermediates formed from AN-1 are in an even more complex, nonlinear and possibly highly branched form than wild type replicative intermediates (Martinez *et al.*, 1996). HSV-1 replicating DNA cannot migrate from the well on pulsed-field gel electrophoresis (Bataille and Epstein, 1994; Severini *et al.*, 1994; Zhang *et al.*, 1994; Martinez *et al.*, 1996) which suggests that replicative intermediates are complex and very large. Restriction enzymes that cut once per genome length can release some monomeric length linear molecules into the gel in replicating wild type DNA, although the majority of the viral DNA remains in the well. In contrast, in AN-1 infected cells, single cutting restriction enzymes do not release discrete monomer genomes, suggesting that this DNA is even more branched than wild type. It has been suggested that the defect in the AN-1 mutant may be the inability to debranch a genome from the interlinked mass of replicating DNA, resulting in abortive packaging (Shao *et al.*, 1993, Martinez *et al.*, 1996). However, recent evidence has been provided that suggested that AN has no Holliday resolvase activity. In these reports, a mutant was created that lacked exonuclease activity but retained endonuclease activity. This mutant could not complement the defect of AN-1, suggesting that the exonuclease activity alone is required for productive infection (Goldstein and Weller, 1998a). Furthermore, Goldstein and Weller (1998b) were unable to demonstrate resolvase activity with wild type or mutant AN on artificial Holliday structures created from oligonucleotides.

The purpose of this chapter is to determine if there is a viral associated resolvase in HSV-1 infected cells. In this chapter, we show that a nuclease activity is found in partially purified extracts from HSV-1 infected cells that can efficiently digest cruciforms, which are equivalent to Holliday junctions. This nuclease can efficiently

linearize a plasmid with an inverted repeat, which extrudes a cruciform due to negative supercoiling, but not if the same plasmid is relaxed by topoisomerase I. Synthetic cruciforms formed from oligonucleotides are cleaved into linear duplex products. We suggest that AN may be involved in the Holliday resolvase activity because this activity is absent in mock infected or null mutant AN-1 infected cell extracts.

2.2 Materials and Methods:

2.2.1 Cells and viruses

Vero cells (African green monkey kidney) were maintained in minimal essential media (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 4% calf serum, 100U/ml of penicillin and 100 µg/ml of streptomycin (GIBCO BRL). The KOS strain of HSV-1 was used as the wild-type virus . The null mutant AN-1 and the permissive cell line 6-5 were kindly provided by Sandra Weller (University of Connecticut Health Center, Farmington, Connecticut) and were maintained as described (Shao *et al.*, 1993).

2.2.2 Enzymes

The bacteriophage T7 endonuclease I, purified from *Escherichia coli* transformed with a plasmid expressing phage T7 gene 3 as previously described (de Massey *et al.*, 1987), was kindly provided by A.R. Morgan (University of Alberta, Edmonton, Alberta). *E.coli* Ruv C was purified from the overexpressing *E. coli* strain GS1191 (JM101 carrying pGS762) (Sharples and Lloyd, 1991) as previously described (Dunderdale *et al.*, 1991).

Cell extracts were prepared by infecting cells at a multiplicity of 10 plaque forming units per cell. At 15 hrs post infection, the cells were detached with phosphate buffered saline with EDTA (137mM NaCl, 2.7mM KCl, 5mM Na_2HPO_4, 0.7mM KH_2PO_4, 2mM EDTA, pH 7.5) and centrifuged at 1400 x g for 10 minutes. The pelleted cells were resuspended in buffer A (20mM potassium phosphate pH 8.0, 2mM $MgCl_2$, 20% glycerol, 10mM dithiothreitol) and subjected to 3 rounds of freeze and thaw (Hoffmann and Cheng, 1978). Debris was pelleted at 12000 x g for 10 minutes and supernatant was collected. The pelleted cell debris was resuspended again in a small volume of buffer A, subjected to 1 round of freeze and thaw, spun at 12000 x g for 10 minutes, and the supernatant was pooled with the previous supernatant.

Enzymes from the infected cell extracts were partially purified by a one step purification on heparin sepharose CL-6B (Pharmacia Biotech, Uppsala, Sweden) columns. The columns were prepared according to the manufacturers instructions and equilibrated in column buffer (10mM Tris-HCl pH 8.0, 0.1mM EDTA, 20% glycerol, 1mM DTT, 1mM spermidine). The cell extracts were loaded and eluted with a step gradient of column buffer containing 0M, 0.2M, 0.4M, and 1M NaCl. Fractions were collected and tested for exonuclease and endonuclease activities (see below). A nuclease with both endonuclease and exonuclease activities was routinely found in the 0.4M NaCl elution. This single step purification yielded enzyme that was active for approximately 6 months when stored at -20 ^{o}C. Protein concentration was determined by the Bio-Rad (Hercules, CA, USA) protein assay.

2.2.3 Plasmid substrates

Plasmid pSCB-1a is a pUC19 derived plasmid carrying a 1.5 kb inverted repeat from the Shope fibroma virus telomere and was constructed as previously described (DeLange *et al.*, 1986). The plasmid was maintained in the recombination deficient *Escherichia coli* strain DB1256. Relaxation of pSCB-1a to generate the lineform was done using wheat germ topoisomerase I (Promega, Madison, WI, USA) as previously described (Stuart *et al.*, 1992).

2.2.4 Detection of exonuclease activity

To detect exonuclease activity, an ethidium bromide fluorescence assay was employed. This assay is based upon the relative fluorescence emitted from ethidium bromide intercalated into double-stranded DNA (dsDNA), which gives a relative concentration

of dsDNA in a sample. Degradation of DNA would be observed as a drop in relative fluorescence.

Exonuclease reactions were done in a reaction buffer of 50mM Tris-HCl (pH 7.5 or pH 9), 10mM $MgCl_2$ or $MnCl_2$, 1mM DTT, 1mM spermidine and 2.5µg of sonicated calf thymus DNA (Sigma, St. Louis, MO, USA) in a final volume of 50µl . The reaction was initiated by adding partially purified lysate to the reaction mixture on ice followed by incubation at 37°C. At various time points, 10 µl aliquots were removed and the reactions were terminated by dilution of these aliquots into 1.2mL of pH 12 fluorescence buffer (0.5µg/ml ethidium bromide, 20mM K_3HPO_4 pH 11.8, 0.5mM EDTA) (Morgan *et al.*, 1979a). Fluorescence was measured on a Sequoia - Turner (Mountain View, CA, USA) model 450 digital fluorometer with a NB520 excitation filter and SC605 emission filter.

2.2.5 Detection of endonuclease activity

Detection of endonuclease activity is based on an ethidium bromide fluorescence assay as described above. For endonuclease, a supercoiled, covalently closed circular plasmid was used as the substrate. Endonuclease reactions were performed in 50mM Tris-HCl (pH 7.5 or pH 9), 10mM $MgCl_2$ or $MnCl_2$, 1mM DTT, 1mM spermidine, and 2.5µg of pSCB1a in a final volume of 50µl. The reaction was initiated by adding partially purified lysate on ice followed by incubation at 37°C. At various time points, a 10µl aliquot was removed and terminated in pH 12 buffer. Before the fluorescence was determined, the sample was denatured for two minutes at 95°C and quickly cooled to room temperature in a water bath. A nicked plasmid would not be able to re-anneal and a drop in fluorescence would be detected. Fluorescence was measured on a Sequoia-Turner model 450 digital fluorometer with a NB520 excitation filter and SC605 emission filter.

2.2.6 Visualization of products from digestion by nucleases

Digestions with plasmid pSCB1a were performed as described above except that the reaction was terminated by the addition of 2µl of 120mM EDTA, 3% sodium dodecyl sulfate (SDS) to a 10µl aliquot of reaction mixture. The samples were extracted with phenol : chloroform (1:1), ethanol precipitated, and resuspended in 10mM Tris-HCl pH 8, 0.1mM EDTA. The samples were electrophoresed on 0.8% agarose / 0.5XTBE (1X TBE = 89mM Tris borate, 2mM EDTA) gels, stained with ethidium bromide and visualized by UV illumination.

2.2.7 Synthetic Cruciforms

Synthetic cruciforms were prepared by annealing 4 partially complementary oligonucleotides. Oligonucleotides were synthesized on a PCR-mate ABI (Applied Biosystems, Foster City, CA, USA) 391 DNA synthesizer.

Cruciform was created using: oligonucleotide M1, 5'- GTG ACC GTT ACC TTG ACG TTG CAG TCT AGC TAT GAC TG - 3'; oligonucletide M2, 5'- CAG TCA TAG CTA GAC TGC AAC GTC AGT ACG TAG GTA GC - 3'; oligonucleotide M3, 5'- GTC ACC TAC GTA CTG ACG TTG CAG TAC TAT GGA CAA GC - 3'; oligonucleotide M4, 5'- GCT TGT CCA TAG TAC TGC AAC GTC AAG GTA ACG GTC AC - 3'.

Cruciforms were generated by mixing equimolar amounts of each of 4 complementary oligonucleotides in 1XSSC buffer (150mM NaCl, 15mM sodium citrate), incubating at 96°C for 2 min, 65°C for 5min, and then allowing the mixture to cool slowly to room temperature. The mixture was run at 4°C on a 10% nondenaturing polyacrylamide gel with 5mM $MgCl_2$ added. The DNA was recovered

by band excision and elution into 10mM Tris-HCl pH 8, 1mM $MgCl_2$ overnight at room temperature with continuous mixing.

In order to label cruciform at the 3' end, four G residues were added to the 5' terminus of oligonucleotide M1 and designated as M5. Equal amounts of oligonucleotides M5, M2, M3, and M4 were annealed together and gel purified as described above. Labeling was done by filling in the 5' overhang with the Klenow fragment of *E. coli* DNA polymerase I (GIBCO BRL), [α-^{32}P]dCTP (NEN Life Science Products, Boston, MA, USA), and cold dATP, dGTP, and dTTP (Boehringer Mannheim, Mannheim, Germany) (Sambrook *et al.*, 1989). The reaction was stopped by extracting with phenol : chloroform (1:1) and free nucleotides were removed using a NucTrap probe purification column (Stratagene, La Jolla, CA, USA). The labeled cruciforms were ethanol precipitated and resuspended in enzyme reaction buffer (50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 1mM DTT, 1mM spermidine).

Reactions with synthetic cruciforms were performed in the same reaction buffer as above with either 1µg of unlabeled cruciform or 500ng of labeled cruciform in a final volume of 50µl. The reactions were initiated by the addition of enzyme on ice and incubated at 37°C. A 10µl aliquot was removed at various time points and reactions were terminated by adding 1µl of 0.5mg/mL proteinase K, 5%SDS, 0.1M Tris-HCl pH 8.0 and incubated for 10 minutes at 37°C. Samples were electrophoresed on 10% nondenaturing polyacrylamide / 1XTBE gels. Reaction products from unlabeled cruciform digestion were visualized by staining with ethidium bromide and illumination with UV. Reaction products from labeled cruciform digestion were visualized by autoradiography.

2.3 Results

2.3.1 Detection of nuclease activity in partially purified crude extracts

To detect nuclease activity, we employed a quantitative ethidium bromide fluorescence assay. Ethidium bromide will intercalate between the strands of double stranded DNA (dsDNA). The amount of fluorescence is relative to the amount of dsDNA present in a sample. Nuclease digestion of the DNA causes a reduction in the amount of ethidium bromide intercalated DNA and would be seen as a drop in relative fluorescence. To detect exonuclease activity linear DNA is used because it has free ends. For endonuclease detection, a covalently closed circular plasmid is used. After digestion by an endonuclease, the sample is denatured and cooled quickly. If the plasmid is nicked or a dsDNA break is formed by an endonuclease, the two DNA strands would not be able to anneal and will result in a drop in fluorescence. The two DNA strands from plasmid that has not been nicked remain linked together so that they can re-anneal and there will be no drop in fluorescence.

The purification of the nuclease on the heparin-sepharose column was monitored by measuring the exonuclease activity on sonicated calf thymus DNA as a substrate. Figure 2.1A shows the results of the assay on the fraction from the 0.4M NaCl elution. In the fractions collected from the partial purification of HSV-1 infected cell lysate, a drop in fluorescence is observed, particularily in fractions 6-12. This drop in fluorescence is not observed in the fractions from the partial purification of uninfected cells. Therefore, a novel nuclease activity is detected only in HSV-1 infected cells.

In order to determine that the partially purified crude extract has Holliday resolvase activity, it is necessary to demonstrate that the nuclease activity is able to specifically cleave a Holliday junction. The plasmid pSCB-1a (DeLange *et al.*, 1986; Stuart *et al.*, 1992) was used as a model substrate. The 1.5 kb inverted repeat from the

Figure 2.1 Assays of exonuclease and endonuclease activity from partially purified crude extract from HSV-1 infected cells. (A) Crude extracts prepared from wild type HSV-1 (KOS strain) infected and uninfected Vero cells were purified on a heparin sepharose CL-6B column as described in the materials and methods. A 5µl aliquot from each fraction collected from the 0.4M NaCl elution step was tested for exonuclease activity at pH 9 against 0.5µg of calf thymus DNA. The samples were incubated for 30 minutes at 37°C before termination. The solid circles represent fractions from KOS and open boxes represent fractions from uninfected cells (B) A 5µl aliquot from each fraction collected from the 0.4M NaCl elution in fig 1A was tested for endonuclease activity at pH 7.5 against 0.5µg pSCB1a for 30 minutes at 37°C before termination. The samples were denatured at 95°C and cooled quickly before fluorescence was measured. The solid circles represent fractions from KOS and the open boxes represent fractions from uninfected cells.

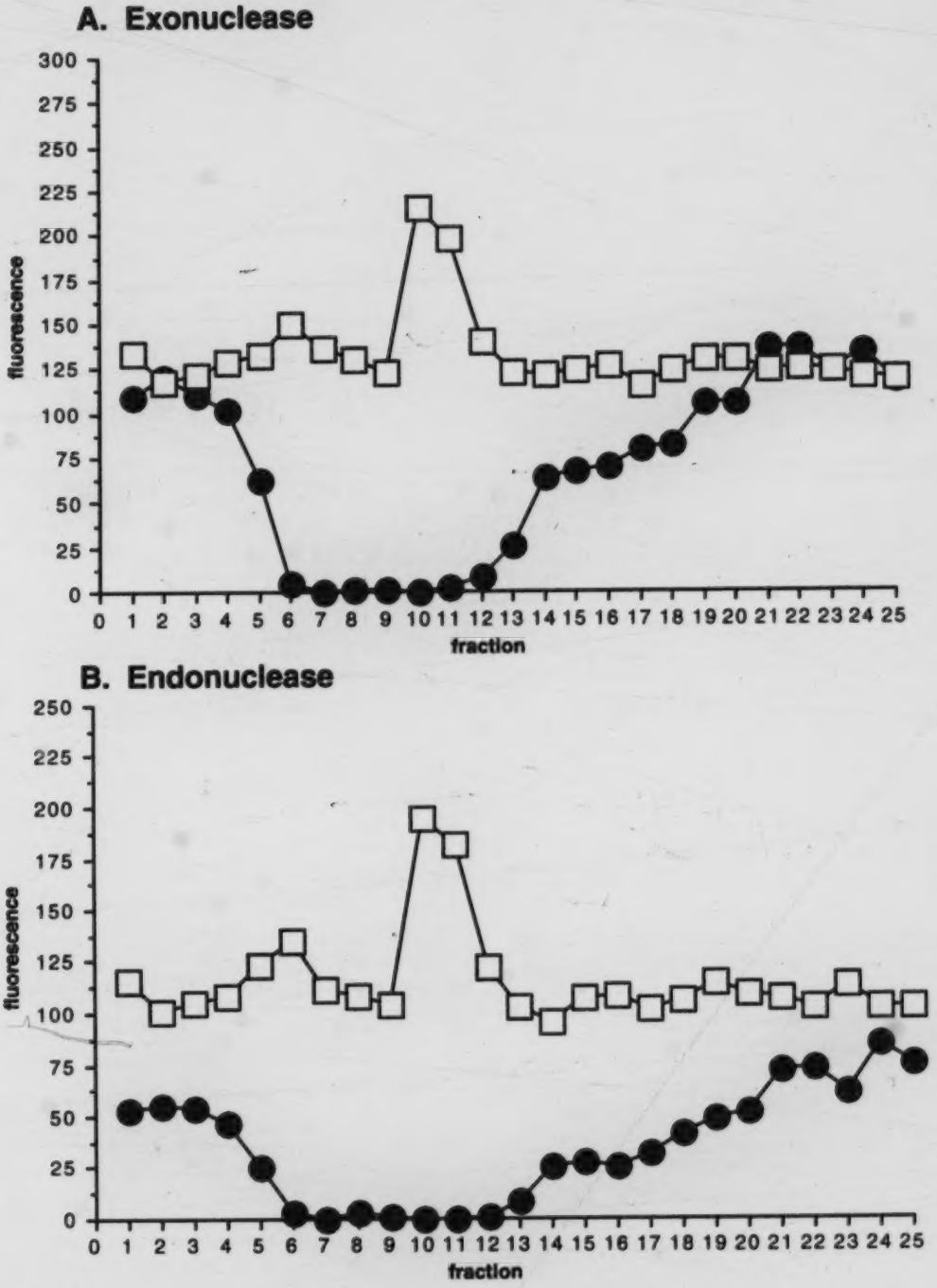
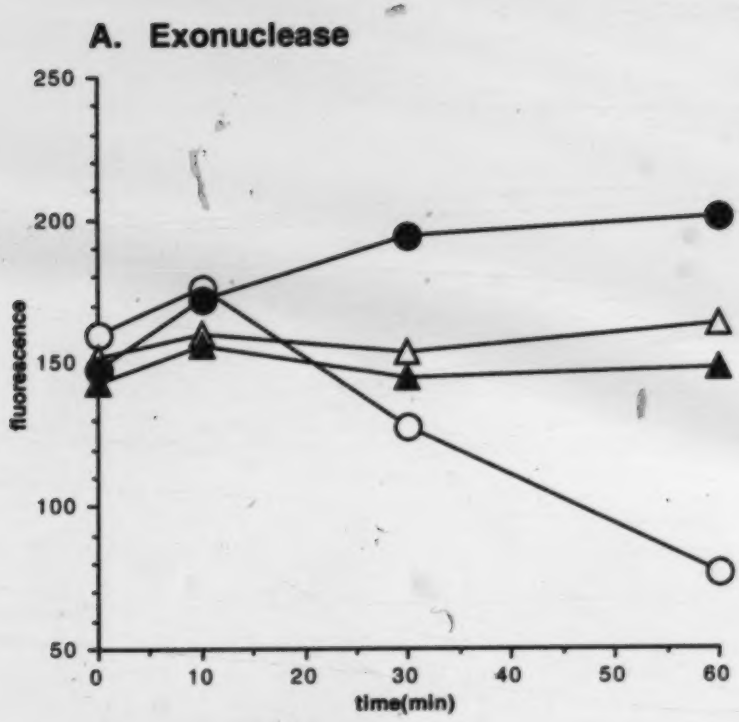
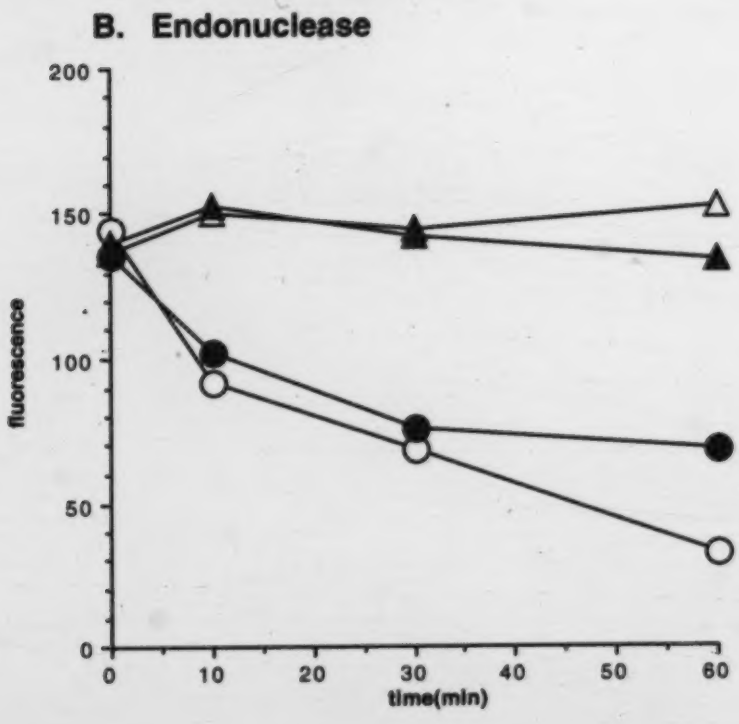

A. Exonuclease
fluorescence
fraction
B. Endonuclease
fluorescence
fraction

Figure 2.2 Nuclease activity in pooled crude extracts. (A) Fractions 6 to 12 from the 0.4M NaCl elution from partial purification of KOS infected (circles) and uninfected Vero cells (triangles) (see fig 2.1) were pooled and tested for exonuclease activity in the presence of $MgCl_2$ (open) and $MnCl_2$ (solid). A 5µl aliquot of pooled enzyme was used in a reaction with 2.5µg of calf thymus DNA in pH 7.5 reaction buffer (see materials and methods) and aliquots were sampled at the indicated times during incubation at 37°C and terminated. (B) A 5µl aliquot of pooled fractions from KOS infected (circles) and uninfected Vero cells (triangles) was tested for endonuclease activity at pH 7.5 against 2.5µg of cruciform plasmid in the presence of either $MgCl_2$ (open) or $MnCl_2$ (solid). Aliquots were sampled at the indicated times during incubation at 37°C and terminated.

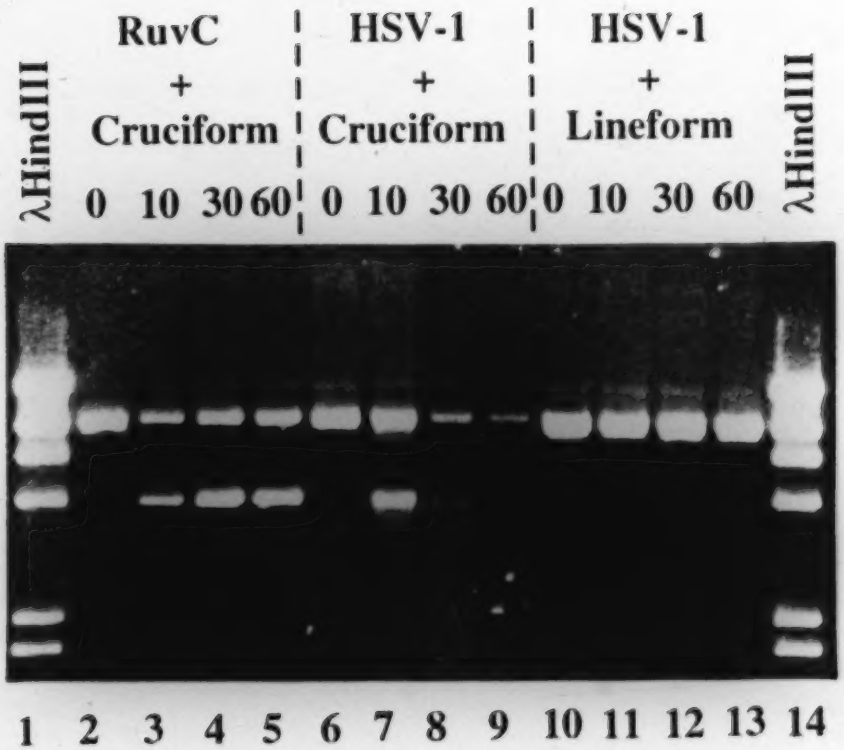

A. Exonuclease
250
200
150
100
50
fluorescence
0
10
20
30
40
50
60
time(min)


B. Endonuclease
200
150
100
50
0
fluorescence
0
10
20
30
40
50
60
time(min)

Shope Fibroma virus telomere will extrude to form the arms of a four stranded cruciform structure when the plasmid is supercoiled (Dickie et al., 1987a). The base of the cruciform is analogous to a Holliday junction. Furthermore, the plasmid can be relaxed by topoisomerase I, which generates the lineform plasmid. The 2 forms of pSCB-1a are identical in sequence but differ in topology. First, the heparin sepharose column fractions were tested in pH 7.5 reaction buffer for ability to cleave cruciform plasmid. After digestion, the samples were denatured and quickly cooled. As seen in fig 2.1B, the same fractions with exonuclease activity (fractions 6-12) also had an endonuclease activity. This activity is absent in fractions from uninfected cells. In figures 2.1A and 2.1B, there was a peak of increased fluorescence in the uninfected cells (fractions 10 and 11). It is likely that this peak was a result of a contaminant that bound to the column co-eluted with the nuclease at 0.4M NaCl. This contaminant could be a substance, such as DNA or protein, that is fluorescent or binds ethidium bromide.

The fractions containing endo-exonuclease activity were pooled (fractions 6-12) and tested again for exonuclease and endonuclease activities. Activity of the pooled nuclease was tested with either MgCl2 or MnCl2 as the cation in the reaction buffer. From the results shown in fig 2.2A, it appears that exonuclease activity in HSV-1 infected cell extracts is present only with MgCl2 and inhibited by MnCl2. Endonuclease activity (fig 2.2B) is present with either MgCl2 or MnCl2. There is no exonuclease or endonuclease activity in the extract from uninfected cells. The selective inhibition of exonuclease but retention of endonuclease activity with MnCl2 is a characteristic of HSV-1 AN (Hoffmann and Cheng, 1978, 1979). It is worth noting that the exonuclease activity in the pooled fractions seen in fig 2.2A is much lower than seen in individual fractions in fig 2.1A. The reactions in fig 2.2A were performed at pH 7.5 whereas the reactions in fig 2.1A were performed at pH 9. The higher

Figure 2.3 Cleavage of pSCB1a cruciform and lineform by nuclease. A reaction buffer at pH 7.5 containing $MgCl_2$ and 0.25µg of either cruciform or lineform plasmid was initiated by the addition of 1µg of protein containing partially purified nuclease. Aliquots were removed at the times indicated and the reaction was terminated, extracted with phenol : chloroform (1:1), ethanol precipitated, resuspended in 10µl of 10mM Tris HCl pH 8, 0.1mM EDTA and run on a 0.8% agarose gel in 0.5XTBE. The gel was stained after with 0.5µg/ml of ethidium bromide and photographed under UV illumination. A reaction using 3ng of *E. coli* RuvC against cruciform plasmid was performed as described above and also run on the gel. The DNA size markers used are λHind III.


λHindIII
RuvC + Cruciform
HSV-1 + Cruciform
HSV-1 + Lineform
λHindIII
0 10 30 60 0 10 30 60 0 10 30 60
1 2 3 4 5 6 7 8 9 10 11 12 13 14

exonuclease activity at an alkaline pH is a feature of HSV-1 AN (Morrison and Kier, 1968; Hoffmann and Cheng, 1978; Strobel-Fidler and Francke, 1980).

A limitation of the fluorescence assay is the inability to show the products of substrate digestion. A nick in only one strand of the plasmid or digestion at the hairpins formed by the extrusion of the inverted repeat would also be detected as endonuclease activity. For an enzyme to be classified as a resolvase, two matched cleavages must be made across the axis of the branch point at the base of the extruded cruciform so that the result of digestion is linear double stranded products.

In order to demonstrate that this was the case for the nuclease from HSV-1 infected cells, the experiment, shown in figure 2.3, was performed. Digestion of the cruciform plasmid by the partially purified nuclease results in production of a 4.2 kb product, which is the size of linearized pSCB1a. Also, the product is the same size as that produced by E. coli RuvC, which is a known resolvase (Dunderdale et al., 1991; Iwasaki et al., 1991). The products from cruciform cleavage with partially purified nuclease cleavage disappear at later times in the reaction. This is presumably due to the strong exonuclease activity that the nuclease possesses which digests the DNA from the free ends generated by endonucleolytic cleavage of the cruciform.

In contrast to cruciform cleavage, the lineform is not digested by the nuclease. The lineform plasmid was created by relaxation of plasmid pSCB1a with topoisomerase I. Relaxation of the cruciform plasmid results in a plasmid without an extruded inverted repeat (no cruciform). Very faint products appear only after 30 minutes of incubation and the extent of degradation is extremely poor compared to the large amount of degradation of cruciform. It appears that the nuclease has specificity for the topology and not the sequence of the plasmid.

2.3.2 Nuclease activity on synthetic Holliday junctions

Figure 2.4 Structure of the synthetic cruciforms. The sequence of the cruciform formed by annealing oligonucleotides M1, M2, M3, and M4. The cruciform has a core of homology ,which is boxed and in bold, that allows the branch point to migrate within this limited region.

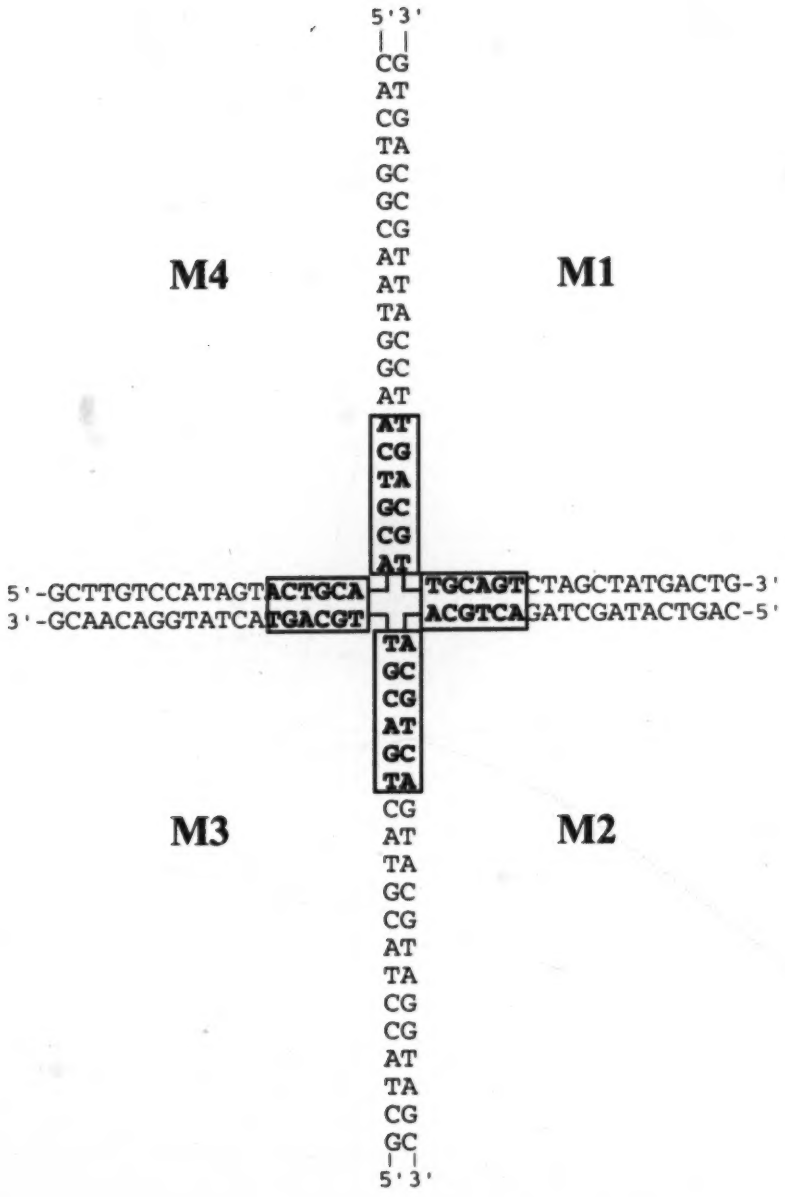
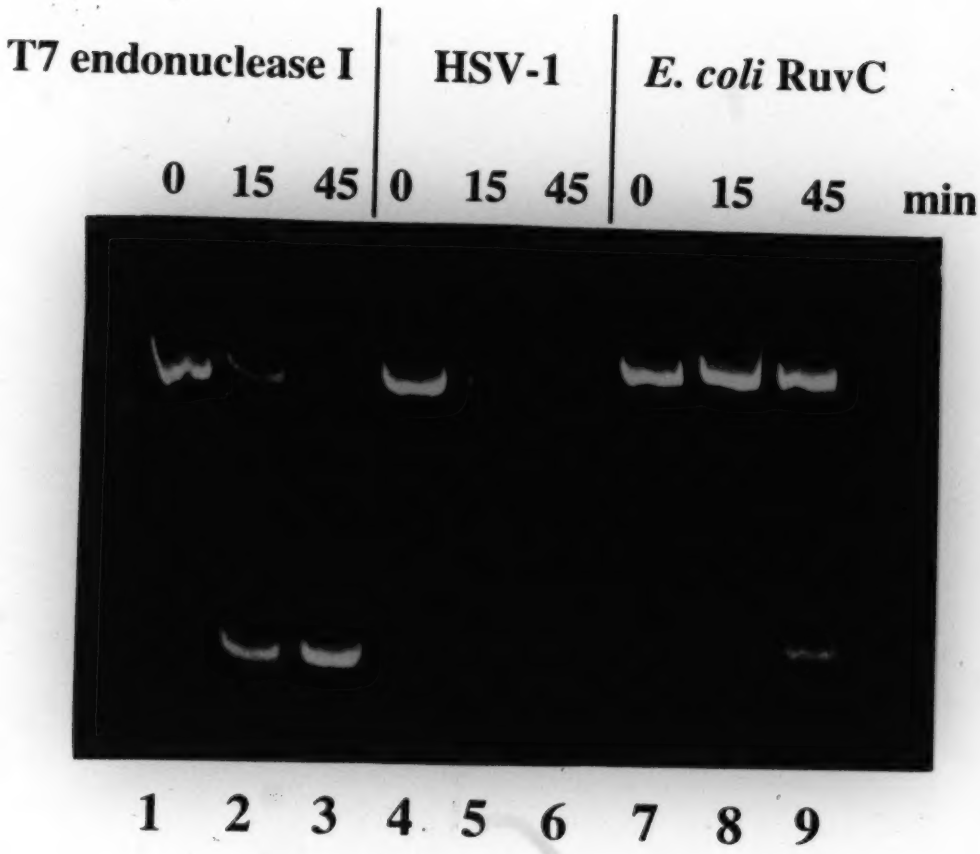

5'3'
M4
M1
5'-GCTTGTCCATAGTACTGCA
3'-GCAACAGGTATCATGACGT
TGCAGTCTAGCTATGACTG-3'
ACGTCAGATCGATACTGAC-5'
M3
M2
5'3'

Figure 2.5 Comparison of digestion of synthetic cruciform by partially purified nuclease from HSV-1 infected cells to known resolvases. Synthetic cruciform was made by annealing oligonucleotides M1, M2, M3, and M4 and was gel purified. Cruciform (1μg per reaction) was digested with 0.15μg of T7 endonuclease I, 0.5μg of protein containing partially purified nuclease from HSV-1 infected cells, and 15ng of *E. coli* RuvC for the times indicated (in minutes). The samples were run on a 10% nondenaturing polyacrylamide gel, stained with ethidium bromide, and photographed under UV illumination.


T7 endonuclease I
HSV-1
E. coli RuvC
0 15 45 0 15 45 0 15 45 min
1 2 3 4 5 6 7 8 9

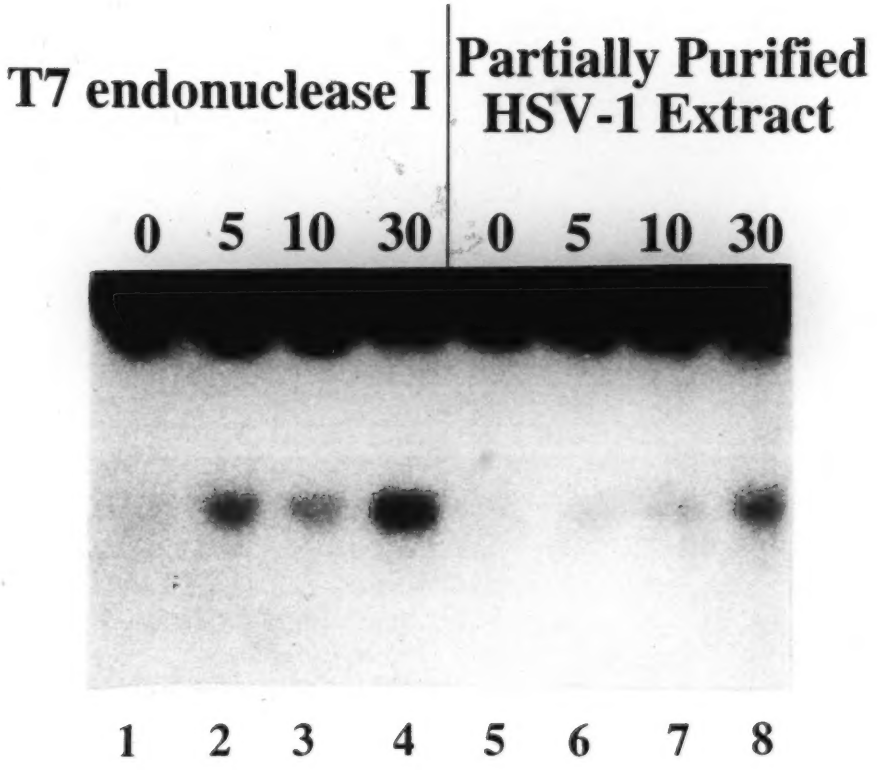
As stated above, one problem with the interpretation of these results from digestion of pSCB1a is that the ends of the cruciform form hairpins. It is possible that the partially purified nuclease has single-stranded DNase activity. Cleavage of the hairpins could also result in a linearization of the cruciform plasmid and lack of the hairpins would explain the inability to cleave lineform. Therefore, synthetic oligonucleotides were used to create a Holliday structure that does not form hairpins.

The synthetic cruciforms were made by annealing 4 oligonucleotides that are partly complementary to each other so that they form a four stranded structure. These cruciforms have the advantage of lacking hairpins or other single stranded regions that could be potential targets (fig 2.4). This cruciform has a central core of homology so that the branch point can migrate. Some resolvases, such as E. coli RuvC, require mobile branchpoints for activity (Dunderdale et al., 1991), whereas others, such as bacteriophage T4 endonuclease VII or bacteriophage T7 endonuclease I, are broader range resolvases that can cleave both fixed and mobile junctions (Dickie et al., 1987b; Mueller et al., 1990; Picksley et al., 1990).

The products of partially purified nuclease digestion of synthetic cruciform were compared to those of digestion by the known resolvases T7 endonuclease I and E. coli RuvC (fig 2.5). The products generated by all three enzymes are identical in size indicating that the nucleases cleave the cruciform into linear duplex products. The products generated from digestion by partially purified nuclease are weak, presumably due to exonuclease activity.

In figure 2.6, a 3'-end labeled cruciform was used in the digestions. The greater sensitivity of radiolabeled cruciform was desired because it allows better visualization of cleavage products that are faint when stained with ethidium bromide (fig. 2.5). We see products accumulating from digestion of cruciform by partially

Figure 2.6 Digestion of 3'-end labeled cruciform. Synthetic cruciform was made by annealing M2, M3, M4, and M5 and was gel purified. The cruciform was 3' end labeled with [α^{32}P]dCTP and the Klenow fragment of *E. coli* DNA polymerase I. The cruciform was digested with either 30ng of T7 endonuclease I or 0.1μg of protein containing partially purified nuclease and aliquots were removed and terminated at the indicated times (in minutes). Each reaction contained approximately 500ng of labeled cruciform. The samples were run on a 10% nondenaturing polyacrylamide gel and autoradiographed on Kodak X-Omat AR film.


T7 endonuclease I
Partially Purified HSV-1 Extract
0 5 10 30 0 5 10 30
1 2 3 4 5 6 7 8

purified nuclease (fig 2.6, lanes 5-8) that are identical in size to the products produced by T7 endonuclease I digestion (fig 2.6, lanes 1-4). We do not see a ladder of bands or a smear from the top to bottom of the gel which would result from exonuclease digestion from the free ends of the cruciform. The results obtained using labeled cruciform (fig 2.6) are similar to those with unlabeled cruciform (fig 2.5). Resolution of a cruciform by a resolvase is a result of nicking the DNA strands across a branch point to generate linear dimer products. Thus, it appears that the nuclease activity partially purified from HSV-1 infected cells digests cruciforms in a manner similar to other known resolvases.

2.3.3 HSV-1 alkaline nuclease is involved in cleavage of a cruciform structure

The nuclease used in this chapter was only partially purified by a single chromatographic step on a heparin-sepharose column. Many proteins of host cell or viral origin would be co-purified with the nuclease The inhibition of exonuclease activity by $MnCl_2$ and greater activity at alkaline pH observed in fig 2.2 are properties of HSV-1 alkaline nuclease. We hypothesized that AN may be involved in the observed resolvase activity. Because the nuclease is in a partially purified state, it was necessary to establish if AN is itself responsible, or is needed, for the observed resolvase activity. However, the novel endo-exonuclease may have been induced by HSV-1 infection. To test the hypothesis that AN is involved, cell extracts were prepared from wild-type KOS infected, mutant AN-1 (Weller *et al.*, 1990) infected, and mock infected Vero cells. AN-1 infection would result in the production of all HSV-1 gene products except for alkaline nuclease and contain any proteins induced by viral infection. All three extracts were subjected to the same partial purification as described in the materials and methods and identical fractions were pooled (fig 2.7).

Figure 2.7 Partial purification of KOS, AN-1, and uninfected Vero cells. Cell extracts from KOS, AN-1, and mock infected Vero cells were partially purified on heparin-sepharose CL-6B and a 5µl aliquot from each fraction from the 0.4M NaCl elution was tested against 0.5µg of pSCB1a for endonuclease activity. The reactions were incubated at 37°C for 30 minutes, denatured and quickly cooled before determination of fluorescence. Fractions 3 and 4 from each purification were pooled for further analysis.

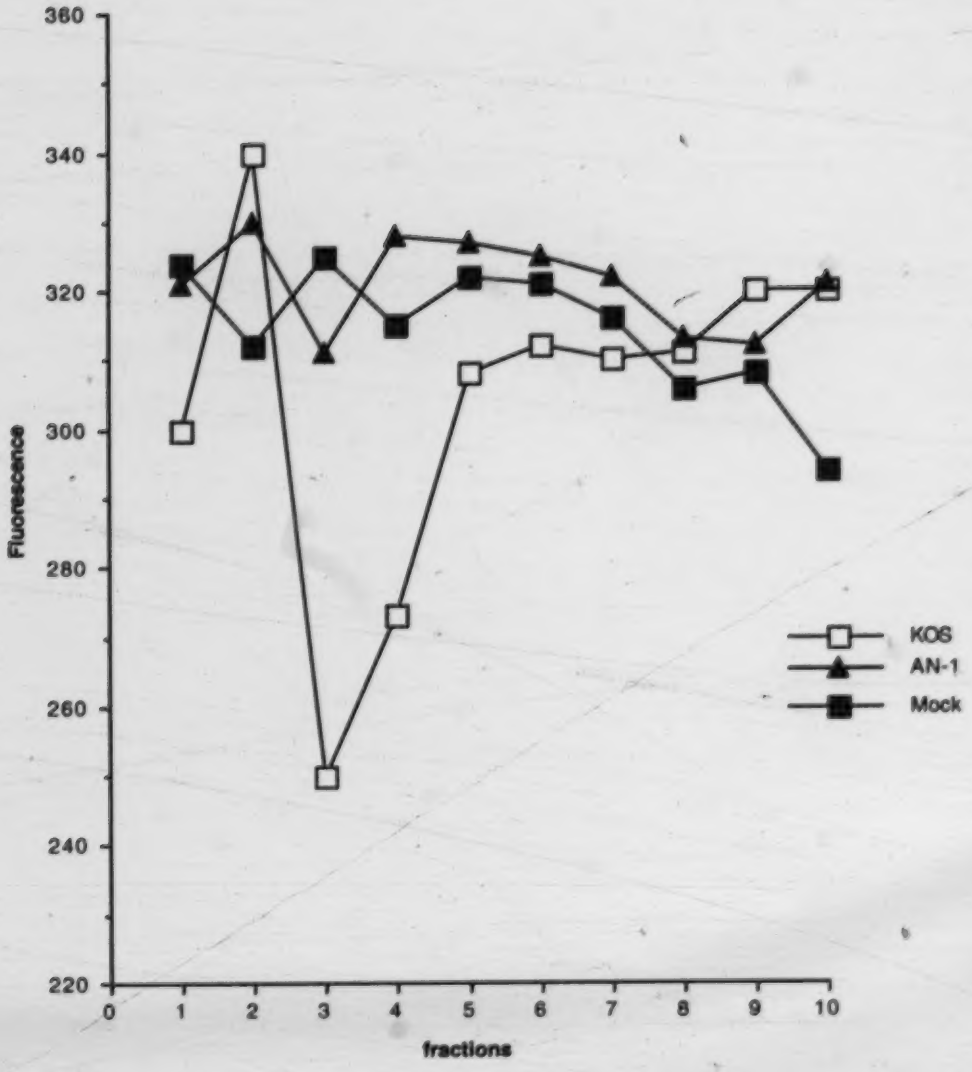
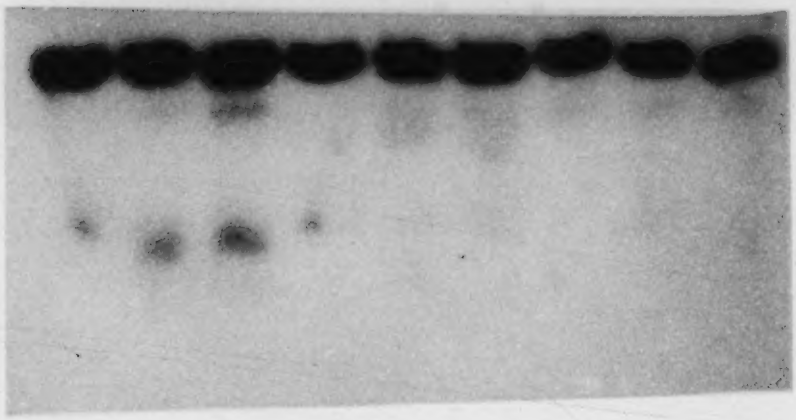

360
340
320
300
280
260
240
220
Fluorescence
0
1
2
3
4
5
6
7
8
9
10
fractions
KOS
AN-1
Mock

Figure 2.8 HSV-1 alkaline nuclease is needed for resolvase activity. Pooled fractions from the partial purification of KOS infected, AN-1 infected, and uninfected Vero cells were tested against 500ng of 3' end labeled cruciform for cleavage. Aliquots were removed at the indicated times, terminated, run on a 10% nondenaturing polyacrylamide gel and subjected to autoradiography.


KOS
AN-1
Mock
0 10 20 0 10 20 0 10 20 min
1 2 3 4 5 6 7 8 9

The heparin sepharose purified fractions from the AN-1 infected and mock infected cells did not demonstrate any endonuclease activity. The specific cleavage of mobile cruciform was seen in the KOS fractions, but absent in the AN-1 and mock infected fractions (fig 2.9). Therefore, it is most likely that HSV-1 AN is necessary for the resolvase activity that I observed in the partially purified extract from KOS infected cells.

2.4 Discussion

Holliday junction resolvases are nucleases that are selective for DNA containing branchpoints, especially four way junctions (White *et al.*, 1997). They have been identified from sources ranging from bacteriophages (Mizuuchi *et al.*, 1982; Lilley and Kemper, 1984; Kemper *et al.*, 1984; de Massey *et al.*, 1987; Dickie *et al.*, 1987b), to bacteria (Connolly *et al.*, 1991; Dunderdale *et al.*, 1991, 1994; Iwasaki *et al.*, 1991; Sharples and Lloyd, 1991), yeast (West and Korner, 1985; Symington and Kolodner, 1985; Evans and Kolodner, 1987; Parsons and West, 1988; Kleff *et al.*, 1992; Jensch *et al.*, 1989; Kupfer and Kemper, 1996; Oram *et al.*, 1998; Whitby and Dixon, 1997, 1998), mammalian cells (Jeyaseelan and Shanmugam, 1988; Waldman and Liskay, 1988; Elborough and West, 1990; Couture and Chow, 1992; Hyde *et al.*, 1994) and viruses (Stuart *et al.*, 1992; Garcia *et al.*, 2000). We report that a Holliday junction resolving activity can be detected in HSV-1 infected cells. Holliday junctions are the key intermediate formed during homologous recombination (Holliday, 1964) and resolvases have the ability to specifically recognize and cleave Holliday junctions. Using models of Holliday junctions as substrates, we have demonstrated that a partially purified extract from HSV-1 infected cells that possesses both endonuclease and exonuclease activities is able to cleave these structures in a manner that is consistent with a Holliday resolvase. Inverted repeats in a supercoiled plasmid will be extruded to form the arms of a cruciform. This four-stranded structure is a model of a Holliday junction. The cruciform can be removed by relaxing the plasmid with topoisomerase I generating the lineform plasmid (DeLange *et al.*, 1986; Stuart *et al.*, 1992). The extract from HSV-1 infected cells was able to efficiently cleave the cruciform plasmid which creates free ends that act as substrates for exonuclease. The lineform plasmid, however, is not cleaved. This result suggests that the partially purified nuclease is not sequence specific but may recognize DNA conformation. A problem with these results

is that the exact site of cleavage is not known. For correct resolution of the cruciform, there must be symmetrical cuts across the axis of the branchpoint to form linear products. A double stranded break at any point in the plasmid would produce a linear product.

Other targets that Holliday resolvases have been shown to recognize are synthetic cruciforms formed from oligonucleotides (Dickie *et al.*, 1987b; Elborough and West, 1990; Picksley *et al.*, 1990; Dunderdale *et al.*, 1991; Pottmeyer and Kemper, 1992; Bennett *et al.*, 1993; Garcia *et al.*, 2000). Digestion of the mobile synthetic cruciforms with the partially purified nuclease produces products that are the same size as those created by known resolvases. This suggests that the partially purified nuclease may cleave this substrate in a similar manner to a legitimate Holliday resolvase.

Because the extract used is only partially purified, we cannot say whether this activity is conferred by HSV-1 proteins or is induced from the host in response to HSV-1 infection. We do know that this nuclease activity is not found in mock infected cells. We speculate that HSV-1 AN may be involved in the observed resolvase activity. HSV-1 AN is the product of the UL12 open reading frame and encodes for a 626 amino acid product with and apparent molecular weight of approximately 85kDa (Banks *et al.*, 1983). It possesses both exonuclease and endonuclease activity with a high pH optimum (Morrison and Keir, 1968; Hoffmann and Cheng, 1978, 1979; Strobel-Fidler and Francke, 1980, Banks *et al.*, 1983). A null mutant, called AN-1, was found to synthesize near wild type levels of DNA, but was severely reduced in the production of infectious virions (Weller *et al.*, 1990). Also, this mutant produces an unusually high number of A capsids which arise during abortive packaging (Shao *et al.*, 1993). During DNA replication, the DNA becomes highly branched (Severini *et al.*, 1996). In AN-1, it is believed that the DNA is in an even more complex branched

structure than the DNA of wild type virus (Martinez *et al.*, 1996). It was speculated that AN is required to remove these replication or recombination intermediates prior to or during packaging and failure to due so would result in abortive packaging (Shao *et al.*, 1993; Martinez *et al.*, 1996). The nuclease activity of the partially purified extract and its possible role in resolution of branched DNA led us to believe this nuclease activity may be attributable to AN. Initial assays of the partially purified nuclease by the fluorescence assays revealed that activity was greater at higher pH and that the exonuclease activity was inhibited by $MnCl_2$, properties of AN. Furthermore, we observed that partially purified extract from AN-1 infected cells did not cleave synthetic cruciforms. Since AN-1 produces all the gene products of wild type HSV-1 except AN, we believe that HSV-1 AN is necessary for the observed Holliday resolvase activity found in infected cells.

Recently, it was shown that AN does not possess any Holliday resolvase activity on synthetic cruciforms (Goldstein and Weller, 1998b). These studies were done with purified AN expressed in baculovirus. Since our preparation is partially purified, it is possible that AN could work in concert with other viral or host proteins to generate resolvase activity. This phenomenon is not unprecedented in HSV-1. For example, the VP16 protein is an important transactivator that interacts with host cell protein HCF-1 for transport to the nucleus and subsequently with Oct-1 to bind to DNA (Kristie *et al.*, 1989; Stern *et al.*, 1989; Kristie and Sharp, 1990; Xiao and Capone, 1990; Wilson *et al.*, 1997; La Boissiere *et al.*, 1999). The *E. coli* resolvase RuvC works in concert with RuvA and RuvB proteins. RuvA and RuvB promote branch migration while RuvC is responsible for cleaving of Holliday junctions (Taylor, 1992; West, 1994). There is evidence that AN and the UL29 single stranded binding protein may interact (Thomas *et al.*, 1992), but the significance of this interaction is unknown.

We have found a Holliday resolvase activity from HSV-1 infected cells and believe that this activity may be linked to HSV-1 AN. To explore this hypothesis, it will be necessary to purify AN to determine how closely Holliday resolvase activity correlates with AN. Mapping of the exact cleavage site(s) on cruciform substrates is necessary to demonstrate the specificity of cleavage. These experiments would clarify the role of AN in the observed Holliday resolvase activity.

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Chapter 3

Heterologously Expressed and Purified Herpes Simplex Virus Type 1 Alkaline Nuclease has Holliday Junction Resolving Activity

3.1 Introduction

Infection by HSV-1 induces the production of a virally encoded nuclease called alkaline nuclease (AN) (Kier and Gold, 1963; Morrison and Keir, 1968). AN is a product of the UL12 open reading frame, which encodes for a 626 amino acid product with an apparent molecular weight of approximately 85kDa (Costa *et al.*, 1983; Draper *et al.*, 1986; McGeoch *et al.*, 1986, 1988; Banks *et al.*, 1983). HSV-1 AN possesses both exonuclease and endonuclease with optimal activity at an alkaline pH (Morrison and Keir, 1968; Hoffmann and Cheng, 1978, 1979; Stobel-Fidler and Francke, 1980; Banks *et al.*, 1983) . A divalent metal cation, either Mg^{2+} or Mn^{2+}, is required for activity. Interestingly, with Mn^{2+} as the divalent cation, exonuclease activity is inhibited (Hoffmann and Cheng, 1978, 1979). AN can degrade both single and double stranded DNA in a 5'-3' fashion while the endonuclease prefers supercoiled substrates (Hoffmann and Cheng, 1978, 1979; Hoffmann, 1981; Knopf and Weisshart, 1990). However, the role of AN in the life cycle of the virus is unclear.

Some key insight into the role of AN has been found in studies of a null mutant called AN-1 (Weller *et al.*, 1990; Shao *et al.*, 1993; Martinez *et al.*, 1996). AN-1 synthesizes near wild type levels of DNA, suggesting that it has no role in DNA replication, but is severely restricted in the production of mature virions. Furthermore, cleavage of genomes and encapsidation of DNA occurs but these capsids are unable to egress from the nucleus into the cytoplasm. Study of the capsid forms present in AN-1

infected cells shows an increased number of A capsids compared with wild type. There are three intracellular capsid forms identified in infected cells (A, B, and C) (Gibson and Roizman, 1972). C capsids contain viral DNA and can mature into infectious virions (Perdue *et al.*, 1976). A and B capsids lack DNA. B capsids are filled with VP22a, a cleaved form of the scaffolding protein and believed to be the precursors to C capsids (Perdue *et al.*, 1976; Newcomb and Brown, 1991). A capsids are empty capsids that are believed to arise from abortive packaging events (Sherman and Bachenheimer, 1988). These observations suggest that the defect of the AN-1 null mutant may be in the processing of viral DNA into capsids.

Replicating viral DNA exists as a complex, highly branched network consisting of Y- and X- branches (Severini *et al.*, 1994, 1996; Zhang *et al.*, 1994). On pulsed field gels, wild-type replicating DNA is unable to migrate from the wells of the gel. Some monomer length DNA will be released from the well when the replicating DNA is digested with a restriction enzyme that cuts once per genome. In AN-1, however, replicating DNA does not release discrete monomers upon digestion with single cutting restriction enzyme, suggesting that AN-1 replicating DNA is even more complex than wild-type (Martinez *et al.*, 1996). This structure poses a problem for packaging in that a monomer length genome must be freed from this complex for proper packaging. It was believed that AN is necessary for resolving these replication intermediates and failure to do so would result in abortive packaging (Shao *et al.*, 1993; Martinez *et al.*, 1996; Severini *et al.*, 1994, 1996).

We have provided evidence that a Holliday resolvase activity can be found in HSV-1 infected cell extracts and have suggested that AN is necessary for this activity (Chapter 2). Holliday resolvase activity is a possible mechanism for resolution of replication intermediates. An example of this is found in the replication of the bacteriophage T4 (Mosig, 1987, 1998). In the late phase of replication, genomic ends

which are formed invade homologous sequences on other genomes creating replication forks. This mode of replication leads to a large branched network of concatemers. Debranching of the genomes is accomplished by the product of the T4 gene 49 called endonuclease VII. T4 endonuclease VII has been found to be a Holliday resolvase (Mizuuchi *et al.*, 1982; Kemper *et al.*, 1984).

However, evidence has been provided that AN may not function as a Holliday resolvase (Goldstein and Weller, 1998a, 1998b). A mutant AN that loses exonuclease activity but retains endonuclease activity cannot complement the defect of AN-1 in nonpermissive cells. This suggests that it is the exonuclease activity, not endonuclease activity (resolvase), that is the only activity of AN *in vivo* (Goldstein and Weller, 1998a). Furthermore, using purified AN, these researchers were unable to demonstrate resolvase activity on synthetic oligonucleotide cruciforms (Goldstein and Weller, 1998b). Based on these results, Goldstein and Weller (1998a, 1998b) concluded that HSV-1 AN is not a resolvase.

In this chapter, I will present direct evidence that AN has Holliday resolvase activity, using heterologously expressed and purified AN. Heterologous expression of HSV-1 AN has been described in rabbit reticulocyte lysates (Henderson *et al.*, 1998), baculovirus infected cells (Kehm *et al.*, 1998a, Goldstein and Weller, 1998a), in mammalian BHK-21 cells (baby hamster kidney) using vaccinia virus or Semliki Forest virus expression systems (Kehm *et al.*, 1998b), and in *Escherichia coli* (Bronstein and Weber, 1996). All of the systems produced recombinant AN that had the same activities as previously reported for AN isolated from HSV-1 infected cells. For our studies, we chose to use the *E. coli* expression system because of the ease of obtaining large amounts of active AN (50-60mg per 400mL culture) (Bronstein and Weber, 1996). *E. coli* -expressed AN was purified without extensive chromatography, was stable upon storage at -70^{o}C and retained activity after repeated freeze/thaw cycles.

E. coli expression of AN resulted in the production of insoluble protein. The method described by Bronstein and Weber (1996), involved denaturing the insoluble aggregates of protein with high concentrations of urea. The protein was then refolded by slow removal of the urea by dialysis. This method is very slow. Therefore, a faster method was developed in which the insoluble protein was first solubilized in the anionic detergent sodium dodecyl sulfate (SDS). The sample is quickly diluted to reduce the concentration of SDS to a low level and the milder nonionic detergent Triton X-100 was added. The exchange of a harsh detergent with a mild detergent resulted in soluble and active AN. An advantage to this method of refolding is that the dilution and detergent exchange took 5 minutes to complete, whereas slow removal of urea took at least 1 week.

In this chapter, using highly purified HSV-1 AN expressed in the *E. coli* system, we will present conclusive evidence that the AN has resolvase activity.

3.2 Materials and Methods

3.2.1 Plasmid constructs

The AN gene (UL12) was amplified from HSV-1 strain KOS genomic DNA by PCR with Pfu polymerase (Stratagene, La Jolla, CA, USA) with the supplied buffer supplemented with 10% DMSO on a MJ Research (Watertown, MA, USA) PTC-100 thermal cycler with an initial denaturation step of 94°C for 2 minutes followed by 30 cycles of 94°C for 2 minutes, 60°C for 2 minutes and 72°C for 2 minutes and ended with a final elongation step of 7 minutes at 72°C. The primers used are 5' - GAATTCATGGAGTCCACGGGAGGCCCA -3' and 5'- CTCGGATCCTC AGCGAGACGACCTCCCCGT- 3' which will add a unique EcoRI and BamHI (indicated by underlined sequences) at the 5' terminal end and 3' terminal end of the alkaline nuclease gene, respectively. The PCR product was inserted between the EcoRI and BamHI sites in pALTER-1 (Promega, Madison, WI, USA) to generate pALAN-1. For expression in *E. coli*, the EcoRI to HindIII fragment isolated from digestion of pALAN-1 was inserted into the EcoRI - HindIII sites of pRSETB (Invitrogen, Carlsbad, CA, USA) to generate pRSETAN.

Plasmids pSCB-1a and pSD19, which are pUC13 derived plasmids that carry a 1.5kb and 280bp inverted repeat from the Shope fibroma virus, respectively, were constructed as previously described (DeLange *et al*, 1986; Dickie *et al.*, 1987a; Stuart *et al.*, 1992). The plasmid pIR was generated by annealing oligonucleotides IR1 (5'CGGAATTCCCCGAGTCCGTCGACTACGCGGCCGCGTAGTCGACGGACTC GGGGGATCCCG - 3') and IR2 (5'CGGGATCCCCCGAGTCCGTCGACT ACGCGGCCGCGTAGTCGACGGACTCGGGGAATTCCG - 3') together generating a double stranded DNA fragment with a EcoRI site at the 5' end, a NotI site at the center of the inverted repeat and a BamHI site at the 3' end (sites are underlined). The fragment was digested with EcoRI and BamHI and inserted into the EcoRI -

BamHI sites in pUC19 to generate pIR. The plasmid pUC(AT), which is a pUC19 plasmid into which 20 AT pairs were inserted between the EcoRI and PstI sites, was obtained from New England Biolabs (Beverly, MA, USA).

3.3.3 Expression and Purification of Alkaline Nuclease

The plasmid pRSETAN, which contains a T7 promoter controlling expression of AN, was transformed into the *Escherichia coli* strain MC1061(pT7pol26) cells for expression (Mertens *et al.*, 1995). The cells were grown in LB medium supplemented with 100μg/mL ampicillin, 25μg/mL kanamycin and 10mM glucose at 37°C to an OD_{600} of 0.6 - 0.7. The production of AN was induced by adding isopropyl -β-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM and incubation was continued at 37°C for another 3 hours.

The induced cells were harvested by centrifugation, washed once in 1/10 culture volume with 10mM Tris-HCl pH 8, 0.1mM EDTA, and centrifuged again. Cells were resuspended in 1/10 culture volume with TSE (20mM Tris-HCl pH 8.0, 0.1M NaCl, 10mM EDTA) with 0.1mM phenylmethylsulfonyl fluoride (PMSF) added. Lysozyme was added to 1mg/mL and incubated at room temperature for 10 min with continuous mixing. The cell suspension was frozen to -70°C and quickly thawed at 37°C. The suspension was sonicated and insoluble material was pelleted at 10,000 x g for 10 minutes. Most of the AN was found in the insoluble fraction in inclusion bodies (fig. 3.1). The insoluble pellet was washed twice with 1/10 culture volume with TSE and the remaining insoluble material was pelleted by centrifugation. The insoluble pellet was solubilized in a small volume of 1%SDS, 10mM DTT, 20mM Tris-HCl pH 8, 0.1mM EDTA, 0.1mM PMSF at room temperature. The solubilized inclusion bodies were quickly diluted with 20mM Tris-HCl pH8, 0.1mM EDTA, 0.5M NaCl, 1mM DTT, 0.1mM PMSF, 2μg/ml aprotinin, 1μg/mL leupeptin, 1μg/mL

pepstatin, and 20% glycerol so that the concentration of SDS was 0.025%. Triton X-100 was added to a final concentration of 1%, incubated for 5 min at room temperature and spun down at 10,000 x g for 10 min. The supernatant contained soluble, active AN.

Expression from pRSETAN results in alkaline nuclease with a N-terminal 6xHis tag that was used for purification on Ni-NTA resin (Qiagen, Hilden, Germany). All purification steps were carried out at 4°C. Solubilized AN was bound to Ni-NTA for 1hr. The resin was washed twice with 10 column volumes with 20mM Tris-HCl pH 8, 0.3M NaCl, 10mM imidazole, 0.1mM PMSF, 5mM β–mercaptoethanol. AN was eluted 4 times with 1 column volume fractions with 20mM Tris-HCl pH 8, 0.15M NaCl, 250mM imidazole, 10mM β-mercaptoethanol, 0.1mM PMSF, 2μg/mL aprotinin, 1μg/mL leupeptin, 1μg/mL pepstatin, 0.1mM EDTA, 20% glycerol. Eluted AN was pooled and dialyzed overnight at 4°C against 20mM Tris-HCl, 0.1mM EDTA, 1mM DTT, 0.1mM PMSF, 20% glycerol. Dialyzed AN was centrifuged to remove any precipitated material and the purified AN was stored at -20°C. Enzyme activity is stable for >1 year. Protein concentrations were determined using the Bio-Rad (Hercules, CA, USA) DC protein assay. Typical yield is up to 50mg/L of culture.

3.2.3 Quantification of nuclease activity assays

The assays for exonuclease and endonuclease activities are modifications of assays as previously described by Bronstein and Weber (1996). Exonuclease activity was measured by quantification of the release of acid-soluble nucleotides from degradation of ^{3}H-labeled *E. coli* DNA (NEN Life Science Products, Boston, MA, USA). The labeled DNA was made single-stranded by boiling for 10 minutes and quickly cooled on ice. The reaction mixture contained 50mM Tris-HCl pH 9, 10mM $MgCl_2$, 1mM DTT, 100μg/mL BSA, 25nCi of denatured ^{3}H-labeled *E. coli* DNA and 0.5μg of

purified AN in a total volume of 100µl. The reaction mixture was incubated at 37°C for 30 minutes and terminated by the addition of 1 mL of ice cold 5% Trichloroacetic acid (TCA) and 0.25 mg/mL BSA, incubated on ice for 10 minutes, and centrifuged for 5 minutes at 15000 x g. A 200 µl aliquot of the soluble fraction was added to 7 mL of Ecolite (ICN Biomedicals, Costa Mesa, CA, USA) scintillation fluid and counted in a Beckman (Fullerton, CA, USA) model 6000TA scintillation counter. One unit of exonuclease activity is defined as the amount, in micrograms, of acid-soluble nucleotides released after 1 minute of incubation at 37°C. Specific activity is defined as units of exonuclease activity per milligram of protein.

Endonuclease activity was measured by a plasmid DNA degradation assay using supercoiled pUC19. The reaction mixture contained 50mM Tris-HCl pH 9, 10mM $MnCl_2$, 100 µg/mL gelatin, 1mM DTT, 0.2 µg of supercoiled pUC19 and 0.3 µg of purified AN in a total volume of 25µl. The mixtures were incubated for various times as indicated in figure 3.3 and terminated by the addition of 5 µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol, 0.15M EDTA, 3% SDS). The samples were run on 0.8% agarose / 0.5XTBE gels and stained with ethidium bromide. Reaction rate was determined by measuring the amount of undegraded supercoiled DNA in the gel that was illuminated under UV with a UVP (DiaMed Lab Supplies, Mississauga, ONT, CA) video imaging camera. One unit of endonuclease activity is defined as the amount, in micrograms, of supercoiled plasmid DNA degraded in 1 minute at 37°C. Specific activity is defined as units of endonuclease activity per milligram of protein.

3.2.4 Other proteins

Restriction enzymes HindIII, EcoRI, NotI, and BamHI and DNA modifying enzymes T4 polynucleotide kinase, Taq DNA poymerase, and Klenow fragment of *E. coli* DNA

polymerase I were purchased from Gibco BRL (Gaithersburg, MD, USA). Restriction enzymes AatII and AflIII and T7 endonuclease I were purchased from New England Biolabs (NEB). *E. coli* Ruv C, purified from *E. coli* strain GS1191 (Sharples and Lloyd, 1991) as previously described (Dunderdale *et al.*, 1991), was kindly provided by A.R. Morgan (University of Alberta, Edmonton, Alberta).

3.2.5 Nuclease assays on plasmid cruciforms

Nuclease assays were performed in a buffer containing 50mM Tris-HCl pH 7.5, 1mM DTT, 100µg/mL BSA and 10mM $MgCl_2$ or 10mM $MnCl_2$ as indicated in figure legends. For the time course reactions with pSCB1a, pSD19, pUC19 and pBR322, 1µg of each plasmid was added to reaction buffer with the appropriate cation ($MgCl_2$ or $MnCl_2$) and maintained on ice. The amount of AN added to each reaction was based on the units of endonuclease activity. AN was added and the reactions were initiated by transfer to 37^{O}C. At the indicated times, a 10µl aliquot was removed and the reactions were terminated with addition of 2µl of 150mM EDTA, 3% SDS, 60mM Tris-HCl pH 7.5, 0.3mg/mL proteinase K, incubated at 55^{O}C for 10 minutes, extracted with phenol/chloroform (1:1), and ethanol precipitated. Reactions with RuvC were performed in the same reaction buffer as above but only with $MgCl_2$. For the time course reaction with 1µg pSD19, 12.5ng of ruvC was used in a 50µl reaction . A 10µl aliquot was removed and terminated as above at the indicated times. For pSCB1a, 0.3µg of plasmid and 12.5ng of RuvC was incubated at 37^{O}C for 10 minutes before termination as described above. For the reactions with pIR and pUC(AT), a 20µl reaction contained reaction buffer as above, 0.2µg of each plasmid and 0.005U of AN . Reactions were incubated for 30 minutes at 37^{O}C and terminated by the addition of 1µl of 0.5M EDTA. Samples were run on 0.8% agarose / 0.5XTBE (1X = 89mM Tris borate, 2mM EDTA) gels, stained with ethidium bromide, visualized under UV,

and photographed. Digestions with restriction enzymes or T7 endonuclease I in the above gels contained 0.2μg of each plasmid in the reaction buffer supplied by the manufacturer and incubated for 1 hour at 37^{O}C.

3.2.6 Identification of approximate cleavage sites of plasmid cruciforms

In order to identify the approximate site of cleavage of plasmid pSCB1a DNA by AN, pSCB1a was digested with AN and the digestion was terminated as described above. The samples were subsequently digested with HindIII and electrophoresed on a 0.8% agarose/0.5XTBE gel, stained with ethidium bromide and photographed.

For the plasmid pIR, after digestion by AN, the 5' ends of the cleaved DNA were labeled with [γ-^{32}P] ATP (NEN Life Science Products) and T4 polynucleotide kinase (Sambrook *et al.*, 1989). The samples were digested with either AatII or AflIII, run on a 6% nondenaturing polyacrylamide gel and subjected to autoradiography.

3.2.7 Mapping of the exact cleavage site

The cleavage sites on pIR were mapped by primer extension using the two oligonucleotide primers Fwd (5'-CGGGCCTCTTCGCTATTACG) and Rev (5'CTTTATGCTTCCGGCTCGTATG) which flank the inverted repeat. The primers were 5' end labeled with [γ-^{32}P]ATP (NEN Life Science Products) and T4 polynucleotide kinase. Extension of the primers was performed with Taq DNA polymerase on a MJ Research PTC-100 thermal cycler with an initial denaturation step of 94^{O}C for 2 minutes followed by 35 cycles of 94^{O}C for 30 seconds, 60^{O}C for thirty seconds, 72^{O}C for 90 seconds, and ended with a 5 minute elongation step at 72^{O}C. The samples were analyzed on standard 8% polyacylamide / 7M urea sequencing gels (Sambrook *et al.*, 1989) followed by autoradiography. For comparison, the cleavage

sites of EcoRI, NotI and BamHI digested pIR were analyzed in the same method as for AN digested pIR. The sequences of both strands of pIR were analyzed using the same primers used for the primer extension using a double stranded DNA cycle sequencing kit (Gibco BRL).

3.2.8 Synthetic cruciforms

Cruciforms were made by annealing together 4 partially complementary oligonucleotides to form a four stranded structure as described in chapter 2. For the following experiments, 3' end labeled mobile junction cruciform, created by filling in with [α-32P]dCTP (NEN Life Science Products) and Klenow fragment, was used. Thestandards used for sizing were oligonucleotide M4 (monomer), M1+M4 (dimer), or undigested cruciform. Monomer and dimer standards were labeled with [γ-^{32}P]ATP (NEN Life Science Products) and T4 kinase and cruciform was 3' end labeled with [α^{32}P]dCTP (NEN Life Science Products] and Klenow fragment of *E. coli* DNA polymerase I. Reaction with AN contained 50,000 cpm of labeled cruciform and 0.0025U of AN in the standard reaction buffer used for plasmid digestions except these reactions were supplemented with 1mM spermidine and with $MnCl_2$ as the cation in a final volume of 50µl. Digestions by T7 endonuclease I contained 50,000 cpm of cruciform, and 1 U of T7 endonuclease I in reaction buffer supplied by the manufacturer of T7 endonuclease I (NEB) supplemented with 1mM spermidine in a final volume of 50µl. The reactions were incubated at 37°C and 10µl aliquots were removed at various time points and the reaction terminated with addition of 1µl of 0.5M EDTA. Reaction products and standards were analyzed on 8% nondenaturing polyacrylamide gels and subjected to autoradiography.

3.3 Results

3.3.1 Expression and purification of alkaline nuclease

Characterization of a Holliday resolvase activity presented in chapter 2, was based on using a crude lysate prepared from HSV-1 infected cells. Due to the impurity of this preparation, we could not directly ascribe the Holliday resolvase activity as a function of AN, but could only suggest that AN was involved. In order to more clearly define the activity or role of AN, we needed a purified source. Expression of AN resulted in the production of a large amount of a unique product of approximately 87kDa that is absent in uninduced cells or cells containing the expression vector pRSETB without an insert (fig 3.1). Consistent with the previously published work of Bronstein and Weber (1996), most of the expressed AN in *E. coli* was found in the insoluble fraction, presumably due to aggregation of the expressed protein forming inclusion bodies (Fig 3.1, lane 9).

The expression and purification scheme of Bronstein and Weber (1996) has the advantage over other purification schemes in that no tedious or lengthy chromatographic steps were required. However, to solubilize AN from inclusion bodies, the insoluble fraction, was denatured with 8M urea and an extremely lengthy refolding of AN involving slow removal of the urea took at least 1 week. This lengthy renaturation has been noted as a reason for switching to another expression system such as the baculovirus system used by Goldstein and Weller (1998a). Also, I found that the lengthy refolding process resulted in cleavage of all the expressed protein (data not shown). Therefore, I modified the solubilization and refolding steps to a more rapid method. The insoluble AN was first solubilized in the anionic detergent SDS.

Figure 3.1 Expression of HSV-1 alkaline nuclease. The gene for HSV AN (UL12) was inserted into a pRSETB vector and expressed in *E. coli* MC1061(pT7pol26). Samples were prepared as described in materials and methods and analyzed on a 7.5% polyacrylamide gel stained with Coomassie brilliant blue. The lanes from *E. coli* containing the vector pRSETB alone are titled "B" and the lanes from *E. coli* containing vector with UL12 insert are titled "AN". Uninduced cells are in lanes 1 and 2, IPTG induced cells are in lanes 3 and 4, soluble fraction from induced cells are in lanes 6 and 7, and insoluble fractions from induced cells are in lanes 8 and 9. Lane 5 contains the molecular weight markers and the sizes (in kilodaltons) are shown on the left.

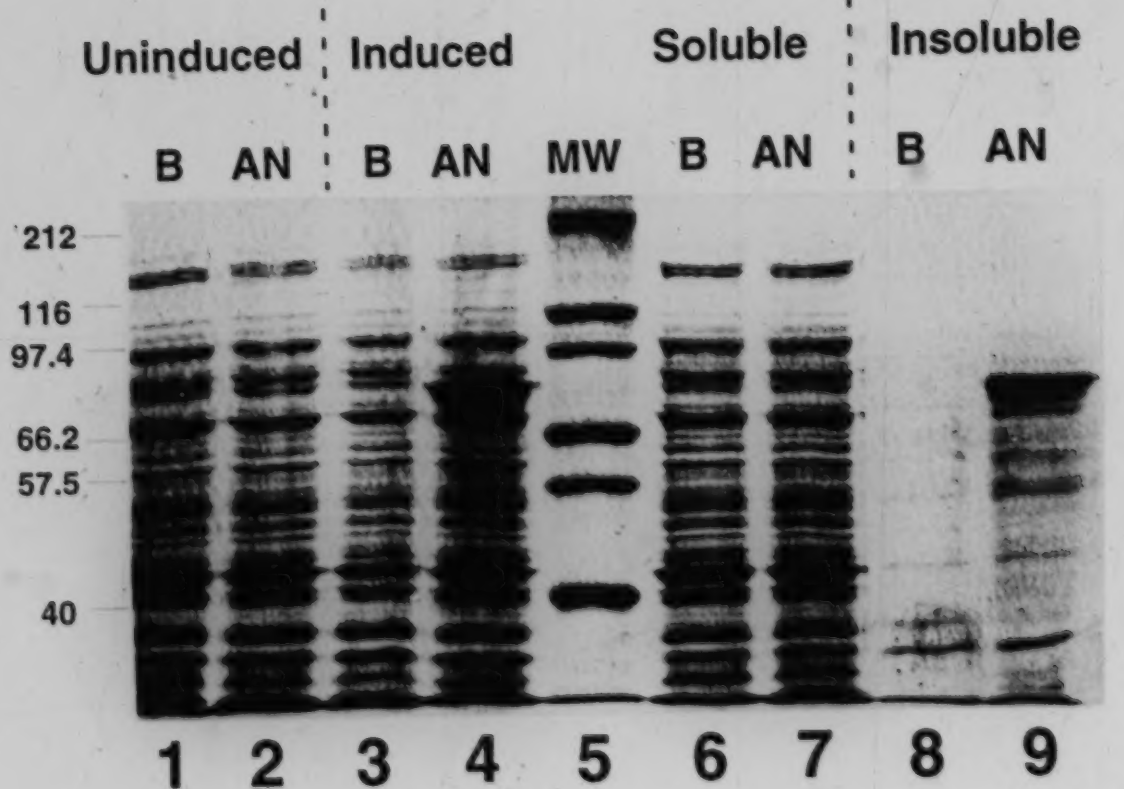

Uninduced
Induced
Soluble
Insoluble
B
AN
B
AN
MW
B
AN
B
AN
212
116
97.4
66.2
57.5
40
1
2
3
4
5
6
7
8
9

Figure 3.2 Purification of alkaline nuclease expressed in *E. coli*.. The detergent solubilized insoluble fraction from expression of HSV-1 alkaline nuclease was purified on Ni-NTA agarose as described in the materials and methods and analyzed on a 7.5% SDS polyacylamide gel stained with Coomassie brilliant blue. Lane 1 is the insoluble fraction of AN before purification, lane 2 is the unbound flow through from the Ni-NTA agarose column, lanes 3 and 4 are subsequent washes of the column, lanes 6-9 are samples from each elution of the column with imidazole, lane 10 is the pooled elutions, and lane 11 is sample after dialysis. The molecular weight markers are in lane 5 and the sizes (in kilodaltons) are indicated on the left.

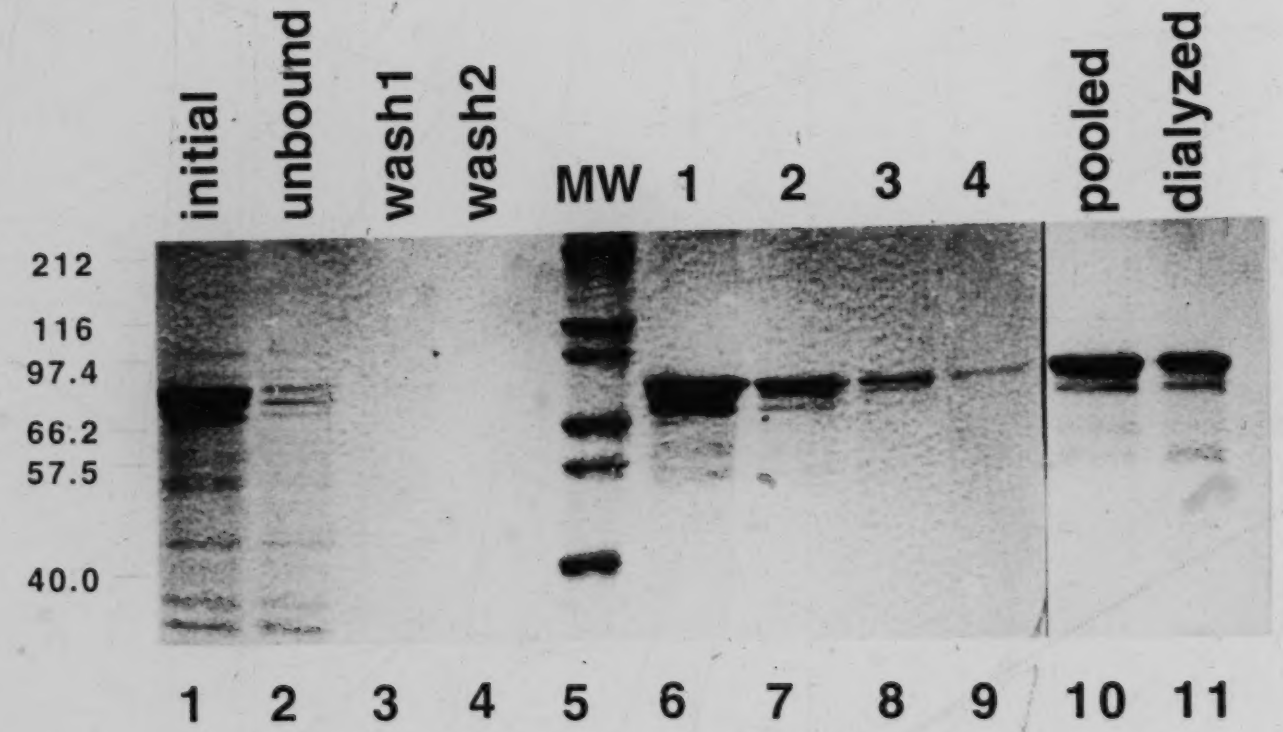
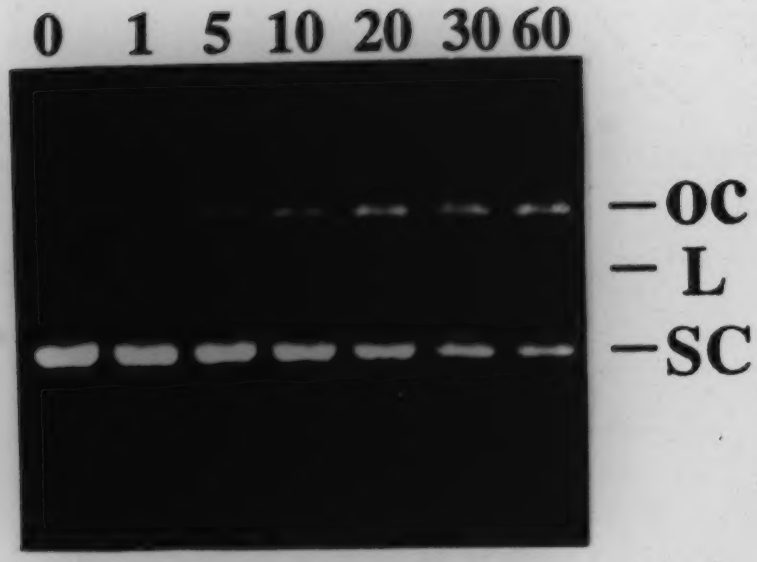

initial
unbound
wash1
wash2
MW
1
2
3
4
pooled
dialyzed
212
116
97.4
66.2
57.5
40.0
1
2
3
4
5
6
7
8
9
10
11

Figure 3.3 Endonuclease assay using supercoiled template. Purified AN (0.3μg) was incubated with 0.2μg of supercoiled pUC19 for various lengths of time as indicated above each lane (in minutes) at pH 9 with $MnCl_2$ reaction buffer to measure endonuclease activity. The digested samples were run on a 0.8% agarose/0.5XTBE gel, stained with ethidium bromide, and the intensity of the supercoiled (SC) band was determined under UV illumination with a UVP video imaging camera. OC indicates the position of open circular plasmid and L indicates the position of linearized pUC19 formed from digestion of SC plasmid.


0 1 5 10 20 30 60
OC
L
SC

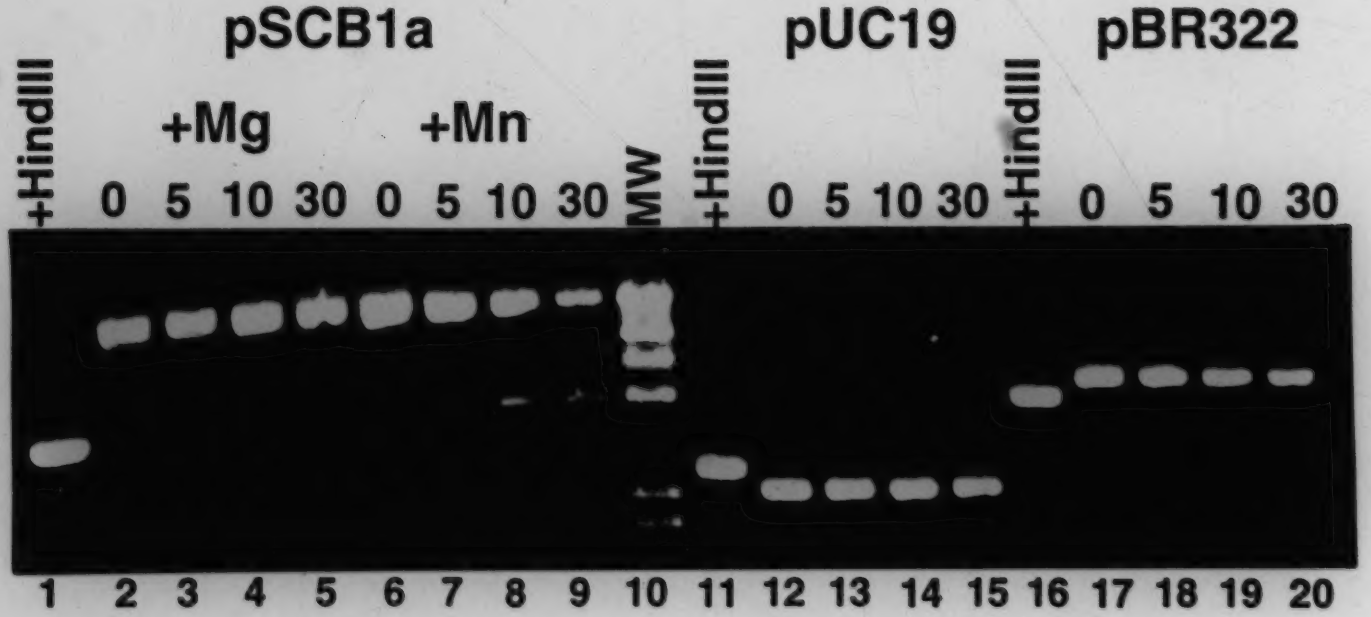
The SDS was diluted and exchanged with the nonionic detergent Triton X-100. SDS is a denaturing detergent and was needed to solubilize insoluble protein. Triton X-100 is a much milder detergent and the exchange of the strong detergent with mild detergent allowed solubilized AN to refold. This detergent exchange method took only 5 minutes, in contrast to more than 1 week by the Bronstein and Weber (1996) method and resulted in soluble and active AN. Since AN was expressed with a N-terminal 6XHis tag, purification was done under native conditions on Ni-NTA agarose to further purify AN (fig 3.2).

3.3.2 Quantification of nuclease activity

Since AN purified from HSV-1 infected cells has previously been shown to have both exonuclease and endonuclease activities (Hoffmann and Cheng, 1978,1979; Strobel-Fidler and Francke, 1980; Banks *et al.*, 1983), we needed to determine that our heterologously expressed and purified AN possessed these activities. The previously reported *E. coli* expressed AN also had exonuclease and endonuclease activities (Bronstein and Weber, 1996). Since the expression, refolding and purification methods used in this chapter are modified from those described by Bronstein and Weber, nuclease was quantified to compare with published results (Bronstein and Weber, 1996)

To determine if exonuclease was present, we used ^{3}H-labeled *E. coli* DNA. Exonuclease activity was measured by the increase in acid-soluble nucleotides after TCA precipitation of the undegraded ^{3}H-labeled *E. coli* DNA. The amount of acid-soluble nucleotides released over time was used to define a unit of activity. We obtained a specific activity of 327.1 U/mg. This value for exonuclease activity compares favorably with the value of 257.6 U/mg reported for AN produced in an *E.coli* expression system (Bronstein and Weber, 1996).

Figure 3.4 Comparison of cleavage of different supercoiled plasmids by purified alkaline nuclease. The plasmids pSCB1a, pUC19, and pBR322 were digested with purified AN. The pSCB1a plasmid has a 1.5kb inverted repeat, whereas pUC19 and pBR322 are just supercoiled plasmids. For each reaction, 1μg of each plasmid was digested by 0.2U of purified AN and aliquots were removed and terminated at the times indicated at the top of the lanes (in minutes). The samples were analyzed on a 0.8% agarose/0.5XTBE gel and stained with ethidium bromide. All the reactions were performed at pH 7.5 and $MgCl_2$, except a reaction with pSCB1a in $MnCl_2$ was also performed. Lanes 1,11,16 are the plasmids linearized with HindIII, lanes 2 -5 are digestion of pSCB1a in $MgCl_2$, lanes 6-9 are digestion of pSCB1a in $MnCl_2$, lanes 12-15 are digestions of pUC19, and lanes 17-20 are digestions of pBR322. Size marker (MW) is λ DNA digested with HindIII (lane 10).


pSCB1a
pUC19
pBR322
+HindIII
+Mg
+Mn
MW
+HindIII
+HindIII
0 5 10 30 0 5 10 30
0 5 10 30
0 5 10 30
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Endonuclease activity was determined by examining the degradation of supercoiled pUC19 and quantifying the intensity of the band of undegraded template remaining after gel electrophoresis (fig 3.3). From this, we determined that AN has a specific activity of 8.3 U/mg. This value is much lower than the previously reported value of 303 U/mg (Bronstein and Weber, 1996). I believe this difference can be attributed to our use of $MnCl_2$ in the assay instead of $MgCl_2$. It is known that the reaction rate of AN in $MnCl_2$ is much lower than in $MgCl_2$ (Hoffmann and Cheng, 1978, 1979). Since we use $MnCl_2$ in most of the digestions to inhibit exonuclease activity, the lower specific activity is representative of the conditions for endonuclease activity used in subsequent experiments.

3.3.3 Cleavage of plasmid cruciforms

Holliday junction endonucleases are structure specific nucleases that recognize and cleave the four-way junctions that arise during recombination and do not depend on specific DNA sequences (Suck, 1997; White *et al.*, 1997). Previous studies in our laboratory have suggested that AN was involved in a Holliday resolvase activity found in HSV-1 infected cell lysates (Chapter 2). Since AN has been shown to possess both strong exonuclease and endonuclease activity, it has been suggested that AN may be responsible for resolving recombination or replication intermediates (Severini *et al.*, 1996) . We used model Holliday structures created from inverted repeats cloned into plasmids. The inverted repeats, under superhelical torsion, extrudes a cruciform. Resolution across the cruciform will produce a linear product. Purified AN was tested for the ability to cleave these plasmids into linear forms.

The ability of purified AN to cleave a plasmid bearing cruciform was tested and the results are presented in figure 3.4. Plasmid pSCB1a contains a 1.5kb inverted repeat derived from the telomere of the Shope fibroma virus (Delange *et al.*, 1986;

Figure 3.5 Cleavage of cruciform plasmid pSD19. Cleavage products from digestion of 1μg of pSD19 (280bp inverted repeat) by 12.5ng of *E. coli* RuvC and 0.2U of AN were analyzed on a 0.8% agarose/0.5XTBE gel stained with ethidium bromide. Reactions were performed at pH 7.5 with $MgCl_2$ for RuvC and at pH 7.5 with either $MgCl_2$ or $MnCl_2$ for AN. The reaction was terminated by removing aliquots at the indicated times (in minutes). Lanes 2-5 are products from RuvC digestion, lanes 6-9 are products from AN digestion in $MgCl_2$, and lanes 10 -13 are products from AN digestion in $MnCl_2$. The size markers are λ DNA digested with HindIII (lane1) and 1kb ladder (lane 14). The arrow on the right indicates position and size of linearized pSD19.

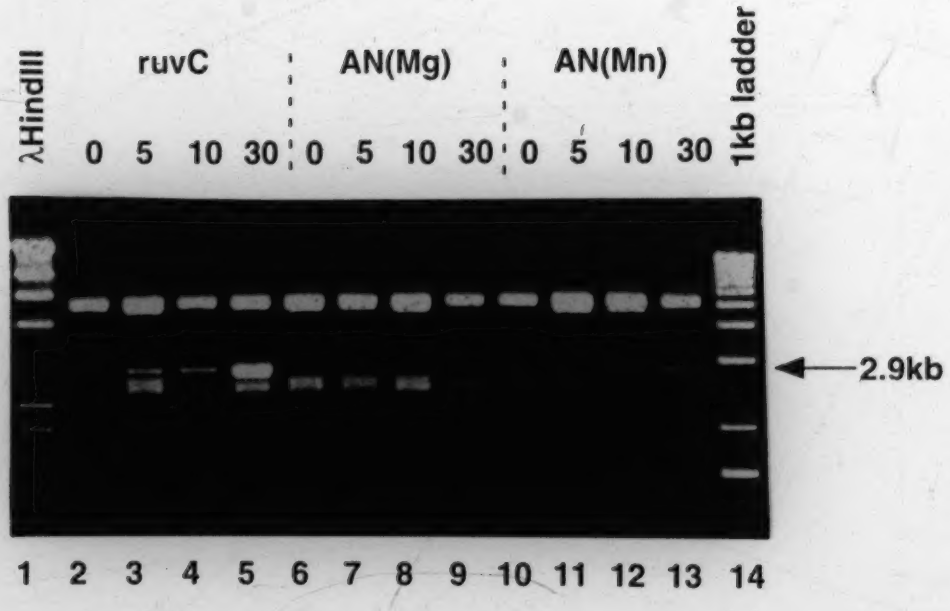
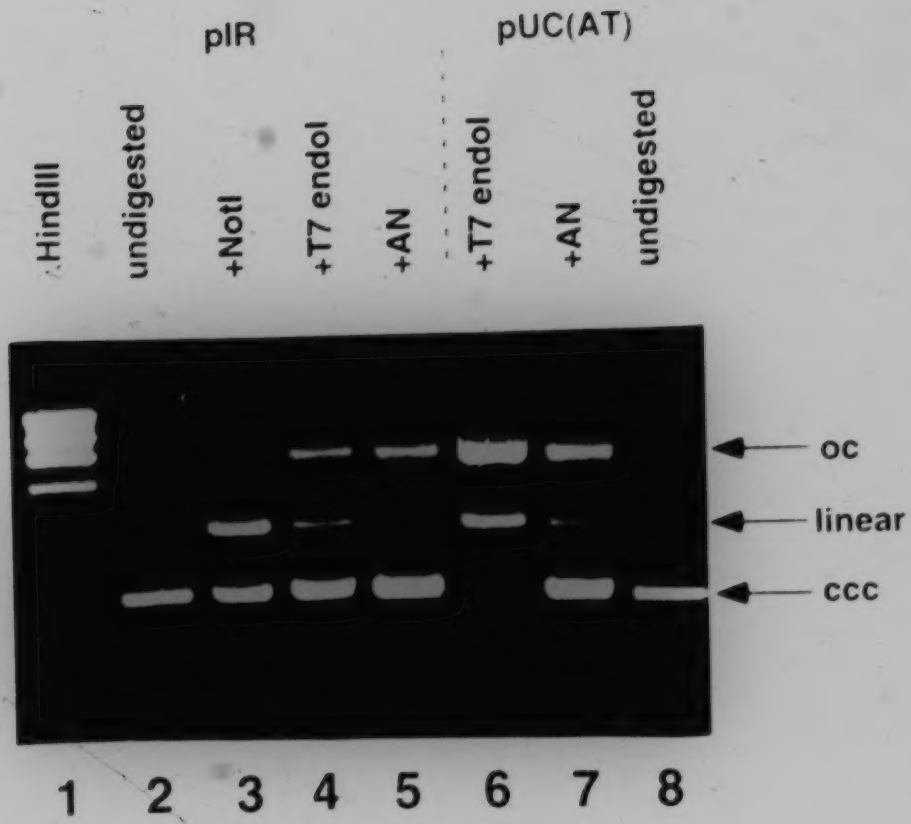

λHindIII
ruvC
AN(Mg)
AN(Mn)
1kb ladder
0 5 10 30 0 5 10 30 0 5 10 30
2.9kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3.6 Cleavage of plasmids pIR and pUC(AT). Plasmid pIR has a 44bp inverted repeat cloned into pUC19 and pUC(AT) has 20 AT pairs cloned into pUC19. In each lane, 0.2μg of plasmid was digested for 30 minutes with 2U of T7 endonuclease I or 0.005U of AN (at pH 7.5 with $MnCl_2$) and analyzed on a 0.8% agarose/0.5XTBE gel and stained with ethidium bromide. Lane 4 shows pIR digested with T7 endonuclease I, lane 5 shows pIR digested with AN, lane 6 shows pUC(AT) digested with T7 endonuclease I and lane 7 shows pUC(AT) digested with AN. Undigested pIR and pUC(AT) are shown in lanes 2 and 8, respectively. Digestion of pIR with NotI shows that approximately 50% of pIR is NotI resistant and therefore has crucifrom extruded (lane 3). Size marker in lane 1 is λ DNA digested with HindIII. The positions of open circular (oc), linear, and closed supercoiled (ccc) forms of the plasmids is indicated on the right.


pIR
pUC(AT)
HindIII
undigested
+NotI
+T7 endol
+AN
+T7 endol
+AN
undigested
oc
linear
ccc
1 2 3 4 5 6 7 8

Stuart *et al.*, 1992). Digestion of pSCB1a results in an accumulation of a product the size of pSCB1a linearized with HindIII. The free ends generated by endonuclease cleavage of pSCB1a are not digested by the exonuclease activity in the presence of $MnCl_2$. The products in the presence of $MnCl_2$ were more visible than the products in the presence of $MgCl_2$ (fig 3.4, lanes 6-9).

Plasmids with different lengths of inverted repeats, and thus various sizes of cruciform, were also tested for linearization by AN. The plasmids used were pSD19, which contains a 280bp inverted repeat from the Shope fibroma virus telomere (Dickie *et al.*, 1987a); pIR which contains a 44bp inverted repeat of a randomly chosen sequence; and pUC(AT), which consists of 20 AT pairs. As illustrated in figure 3.5, digestion of pSD19 by AN produces a 2.9kb fragment, which is the size of linearized plasmid. Again, products of reactions were more visible when $MnCl_2$ was used (fig 3.5, lanes 10-13). Also, the product was the same size as those produced by cleavage of pSD19 by *E. coli* RuvC (Connolly *et al.*, 1991; Dunderdale *et al.*, 1991,1994; Iwasaki *et al.*, 1991; Sharples and Lloyd, 1991; Taylor, 1992; West, 1994), which is a known resolvase (fig 3.5, lanes 2-5). Figure 3.6 shows the results of digestion of pIR and pUC(AT) with purified AN in $MnCl_2$. Digestion resulted in products that are the same size as the products generated by cleavage with phage T7 endonuclease I, another resolvase (deMassey *et al.*, 1987; Dickie *et al.*, 1987b; Picksley *et al.*, 1990). The digestion of pIR by NotI (fig 3.6, lane 3) was included to show how much of the plasmid sample was cruciform. If the cruciform is extruded, the NotI site will be at either end of the hairpins and therefore not digested by NotI (fig 3.10). The amount of undigested plasmid (NotI resistant) is a measure of the amount of cruciform. Digestion of pIR by T7 endonuclease I is much less than the digestion of pUC(AT) (compare lanes 4 and 6 in fig 3.6) because only 50% of pIR is cruciform, whereas nearly all the pUC(AT) is cruciform and thus is completely digested (fig 3.6, lane 6). Thus, it

appears that AN is able to specifically recognize cruciforms of various sizes. Also, the sequences in pSCB1a (fig 3.4) and pSD19 (fig 3.5) are not similar to the sequences in pIR or pUC(AT). Therefore, it is unlikely that AN recognizes a specific sequence but seems to recognize the topology of the plasmids.

The plasmids used have all been generated by cloning an inverted repeat into a pUC background. Therefore, it is possible that AN may recognize a sequence that is common to all pUC plasmids rather than a cruciform. To test this hypothesis, pSCB1a, pUC19, and pBR322 were digested with AN (fig 3.4). Digestion of pSCB1a (cruciform) showed digestion, whereas pUC19 and pBR322 (neither has cruciform) were not digested. This demonstrates that supercoiled plasmids lacking inverted repeats are not as good substrates for AN.

3.3.4 Mapping of the cleavage sites

A double stranded break anywhere in the plasmid would produce a linear plasmid when visualized on an agarose gel. A Holliday resolvase must make opposable nicks across the axis of the branch point of a Holliday junction to form a linear product. In the case of a plasmid cruciform, resolution would produce a linear DNA product with a hairpin at both ends (fig 3.7). In pSCB1a, which has a unique HindIII site at the base of a putative cruciform, digestion with a resolvase should produce 2 unique products from the 4.2kb linear molecule that correspond to a 3.4kb molecule that is full length plasmid lacking one hairpin and a 750bp molecule which is a hairpin fragment (fig 3.7) (Stuart *et al.*, 1992). The results seen in figure 3.8 show that after digestion with AN followed by HindIII digestion, the 3 fragments expected if resolution occurs are produced: 4.2kb band from undigested pSCB1a and the 3.4kb and 750bp bands from resolution by AN (fig 3.8, lanes 4-9). These 3 fragments are also seen in the lane in which RuvC was used (fig 3.8, lane3). Exonuclease activity by AN on the linear

fragment in the presence of $MgCl_2$ is evident in figure 3.8, lanes 4-6 as shown by loss of the 3.4kb and 750bp products with increasing time of digestion. Since exonuclease is inhibited by $MnCl_2$ (Hoffmann and Cheng, 1978, 1979), the 3.4kb and 750bp products can be seen to accumulate (fig 3.8, lanes 7-9).

Mapping was also done on the plasmid pIR to determine the approximate site of cleavage, which should be at the inverted repeat if AN is a Holliday resolvase. To do so, pIR was first digested with AN followed by digestion with either AatII or AflIII, which cleave pIR at sites flanking the inverted repeat (fig 3.9A and 3.9B). The products were labeled and compared to those produced by NotI cleavage. Digestion of pIR by a resolvase results in production of a linear molecule with a hairpin at either end (fig 3.9B). The size of the hairpin in pIR is half the size of the inverted repeat. Since the NotI site is at the center of the inverted repeat (fig 3.9A), digestion by NotI produces a linear molecule with half of the inverted repeat at each end. The resulting products from NotI digestion followed by restriction enzyme digestion by AatII and AflIII should produce fragments that are the same size expected if a Holliday resolvase digested pIR (compare fig 3.9A to 3.9B) The results shown in figure 3.9C show that a major band is produced from AN+AatII and AN+AflIII that is the same size as that produced from NotI+AatII and NotI+AflIII, respectively. These bands are also found in T7 endonuclease I digestion of pIR. This demonstrates that although cleavage may occur at other sites, the majority of cleavage by both T7 endonuclease I and AN is at the inverted repeat.

A problem, as stated above with the interpretation of the mapping done above is that if AN digested at the hairpins and not at the base of the cruciform, the results would be the same as the results with NotI digestion of pIR. The experiment illustrated in figure 3.9 can only show that digestion of the plasmid occurs within the inverted repeat. To resolve this issue, it is necessary to identify the exact site of cleavage to see

if cleavage occurs at the branchpoint of the cruciform, as would be expected of a Holliday resolvase, or at the hairpins like a single-stranded nuclease.

In order to further define the cleavage site(s) present within the inverted repeat of pIR, we employed a method of primer extension using primers that anneal to regions flanking the inverted repeat (fig 3.10). Extension should continue from the primers until there is a nick or break in the DNA strand. The extensions with AN digested DNA was compared to EcoRI, NotI and BamHI digested pIR. From figures 3.11 and 3.12, it is clear that in the lanes made with AN digested pIR, there are 2 major bands that correspond to the EcoRI and BamHI bands in extensions with both Fwd and Rev primers. The EcoRI and BamHI sites are at the branchpoint of the cruciform (fig 3.10). Therefore, it appears that AN makes cleavages at the branchpoint, which is what is expected of a Holliday junction specific resolvase. A doublet of bands is produced in the primer extensions on AN digested pIR with either Fwd or Rev primer. This may be an artifact of using Taq DNA polymerase because Taq DNA polymerase can add a non template single nucleotide to the 3' end of a DNA fragment (Clark, 1988; Hu, 1993). It is not known if both bands are actual cleavage sites.

We also sequenced pIR using the same primers used in the extension reactions in the hope of identifying the exact nucleotide cleaved. A problem with the sequencing reactions is that the sequence of the inverted repeat is extremely compressed. This is likely due to the use of double-stranded DNA for the sequencing reactions. Even at the high temperatures used for the reactions, it appears that the secondary structure formed by cruciform extrusion remained intact. However, the EcoRI and BamHI sites are at the edges of the compressed region so it is likely that these are at the branchpoint of the hairpin and AN cleaves at or within a few nucleotides of the base of the cruciform structure.

Figure 3.7 Experimental design of mapping of cleavage of pSCB1a by a Holliday resolvase. A Holliday resolvase will cleave specifically the cruciform plasmid (top of figure, right side). Cleavage across the axis at the branchpoint will produce a linear DNA molecule with hairpins of 750bp at each end. Digestion at the unique site by HindIII should release 2 products : a 750bp product representing one hairpin and a 3.4kb product representing one hairpin plus the remainder of the plasmid. (Modified from Stuart *et al.*, 1992)

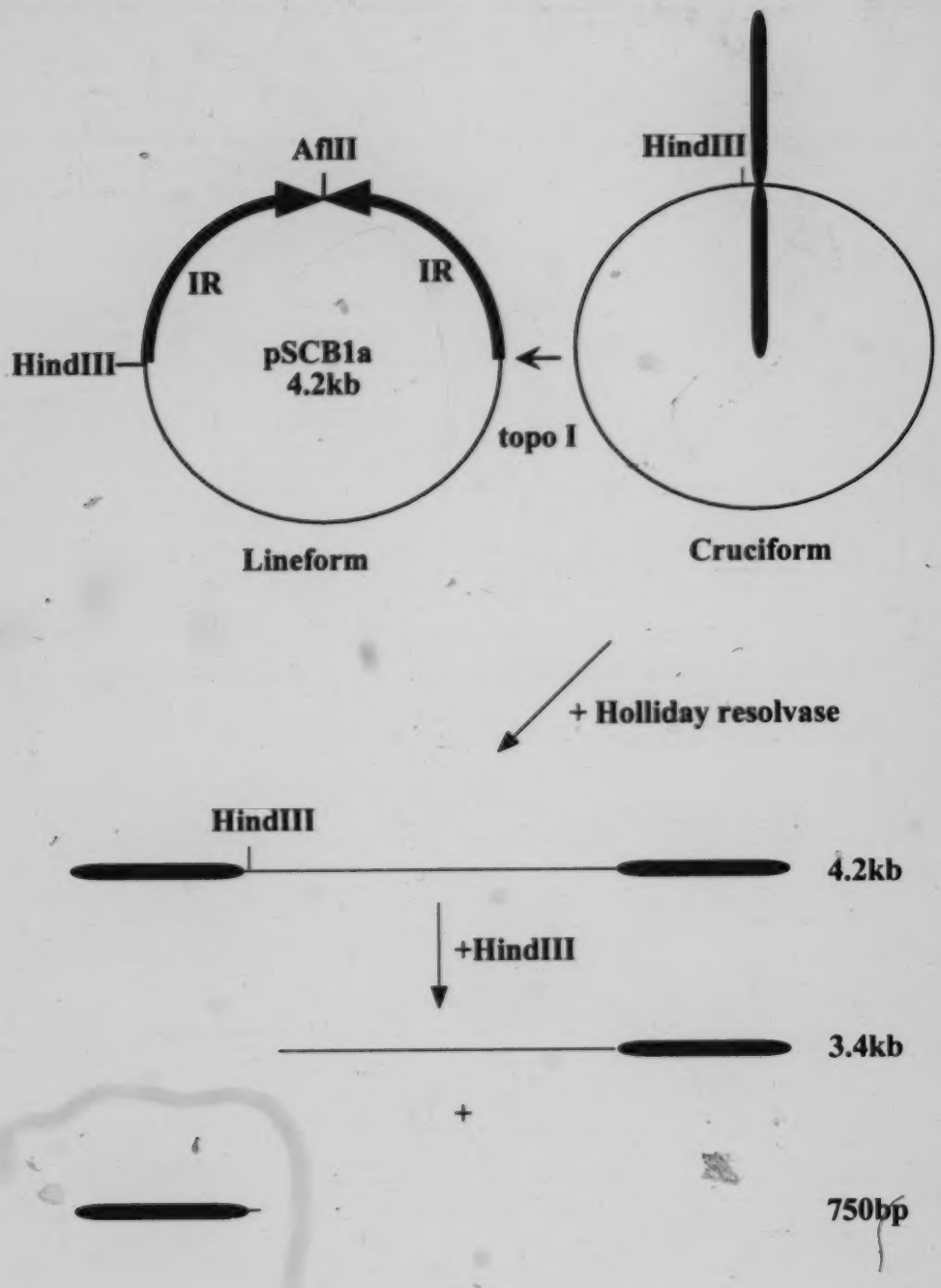

AflII
HindIII
IR
IR
HindIII
pSCB1a
4.2kb
topo I
Lineform
Cruciform
+ Holliday resolvase
HindIII
4.2kb
+HindIII
3.4kb
+
750bp

Figure 3.8 Mapping of cleavage of pSCB1a with alkaline nuclease. The pSCB1a plasmid was digested with *E.coli* RuvC and AN in $MgCl_2$ and $MnCl_2$. For the AN digestions, 1μg of pSCB1a was digested with 0.2U of AN, aliquots were removed at the times indicated in figure (in minutes), and digested with HindIII. For RuvC, 0.3μg of pSCB1a was digested with 12.5ng of RuvC for 30 minutes. The reaction was terminated and the sample was digested with HindIII. Products from the digestions were analyzed on a 0.8% agarose/0.5XTBE gel and stained with ethidium bromide. Lane 2 shows 0.3μg of pSCB1a digested only with HindIII, lane 3 shows RuvC + HindIII digestion, lanes 4-6 show AN in $MgCl_2$ + HindIII digestion and lanes 7-9 show AN in $MnCl_2$ + HindIII digestion. Lane 1 is the size marker of λ DNA digested with HindIII. The location and sizes of the products are indicated on the right.

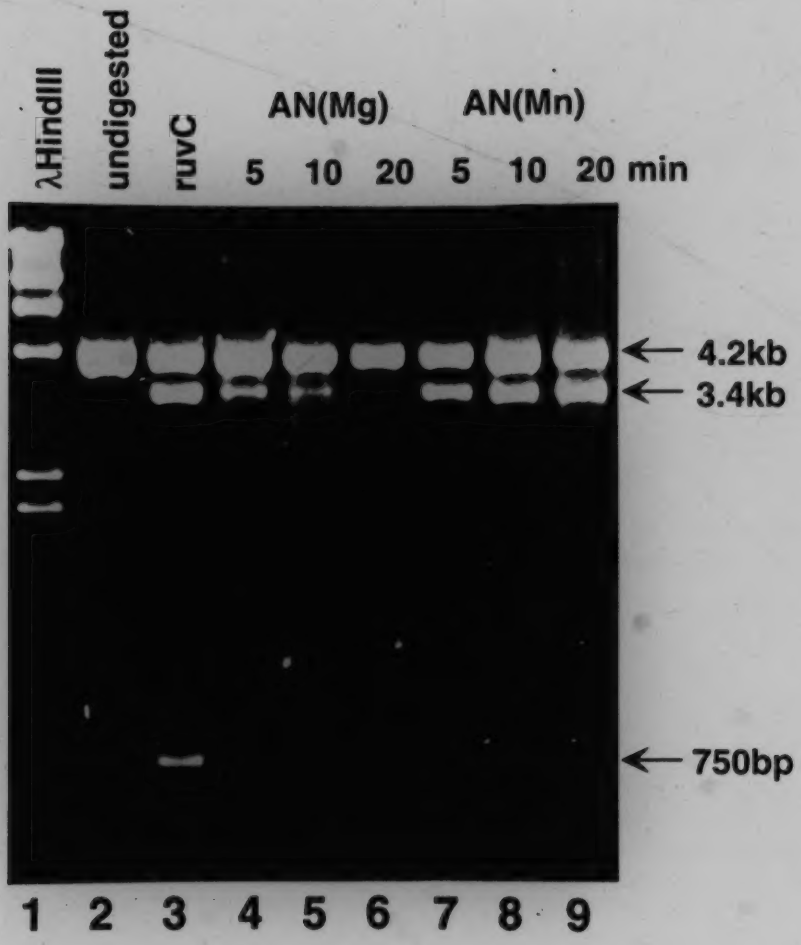

λHindIII
undigested
ruvC
AN(Mg)
AN(Mn)
5 10 20 5 10 20 min
4.2kb
3.4kb
750bp
1 2 3 4 5 6 7 8 9

Figure 3.9 Mapping of cleavage of pIR. (A) Map of the 44bp inverted repeat (IR) region of pIR and the size of fragments generated by restriction enzyme digestion. (B) Results expected from Holliday resolvase digestion followed by restriction enzyme digestion. (C) T7 endonuclease I and HSV-1 AN cleaved pIR was then digested with AatII (lanes 2 and 3) or AflIII (lanes 5 and 6). These products were labeled with [α-^{32}P]ATP and T4 polynucleotide kinase and analyzed on a 6% nondenaturing polyacrylamide gel followed by autoradiography. NotI digested pIR was digested with AatII (lane1) and AflIII (lane 4) and analyzed as above to serve as size markers. The products are indicated by arrows in the figure.

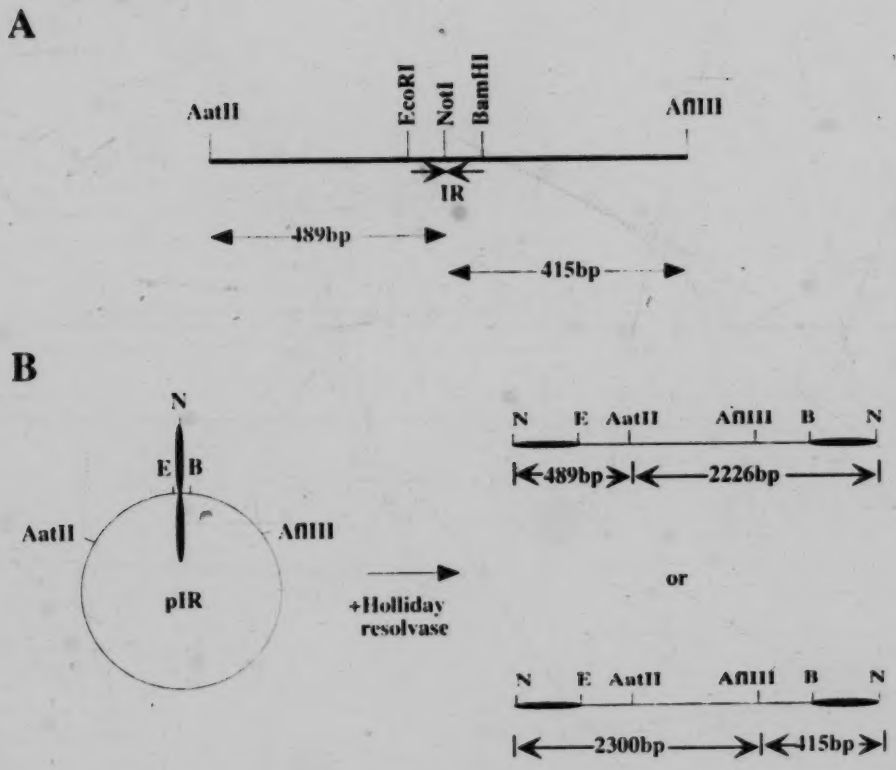

A
AatII
EcoRI
NotI
BamHI
AflIII
IR
489bp
415bp
B
N
E
B
AatII
AflIII
pIR
+Holliday
resolvase
N E AatII AflIII B N
489bp
2226bp
or
N E AatII AflIII B N
2300bp
415bp
C
NotI-AatII
T7endoI-AatII
AN-AatII
NotI-AflIII
T7endoI-AflIII
AN-AflIII
large fragment
489bp
415bp
1 2 3 4 5 6

Figure 3.10 DNA sequence around the inverted repeat of pIR and the sequences of the Fwd and Rev primers are shown. Restriction enzyme sites are boxed and in bold.

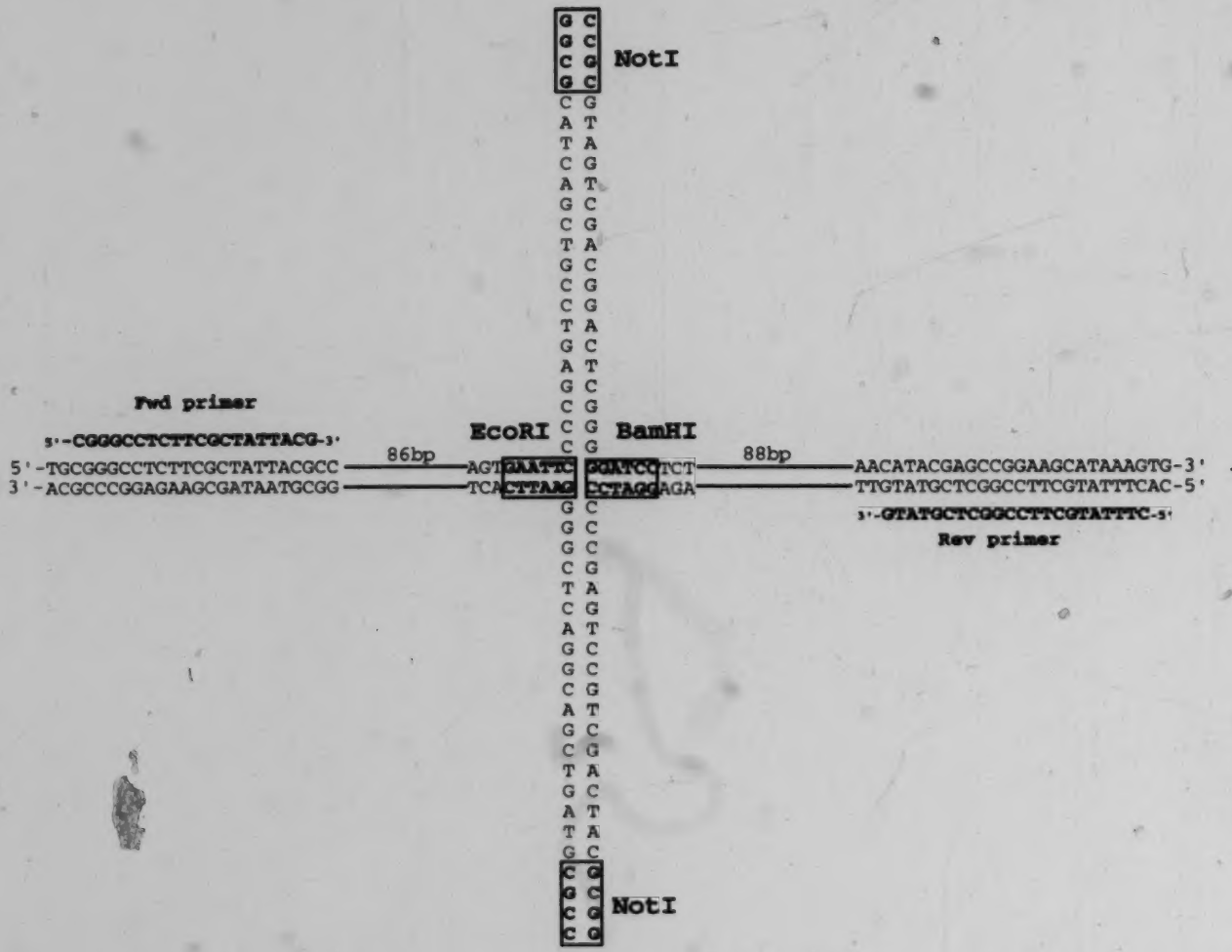

NotI
Fwd primer
5'-CGGGCCTCTTCGCTATTACG-3'
EcoRI
BamHI
86bp
88bp
5'-TGCGGGCCTCTTCGCTATTACGCC
3'-ACGCCCGGAGAAGCGATAATGCGG
AATTC
TCT
AGA
AACATACGAGCCGGAAGCATAAAGTG-3'
TTGTATGCTCGGCCTTCGTATTTCAC-5'
3'-GTATGCTCGGCCTTCGTATTTC-5'
Rev primer
NotI

Figure 3.11 Primer extension analysis of pIR using the Fwd primer. The products from sequencing and primer extension using labeled Fwd primer were analyzed on a 8% polyacrylamide / 7M urea sequencing gel followed by autoradiography. Lanes 1-4 are the sequencing ladder, lanes 5, 6, 7, 8 and 9 are the products from primer extension of undigested pIR, EcoRI, NotI, BamHI, and AN cleaved pIR, respectively.

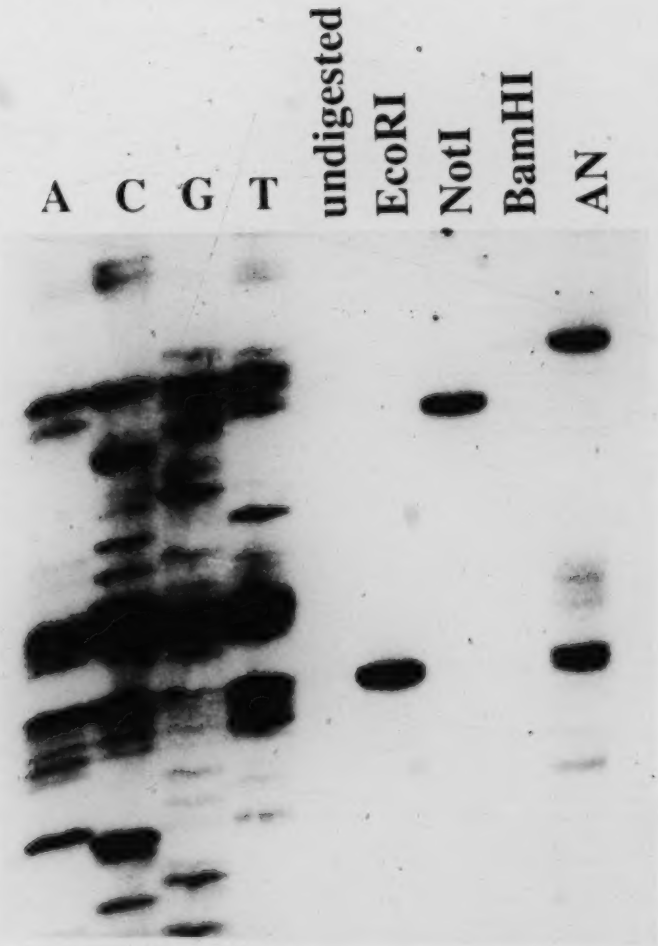
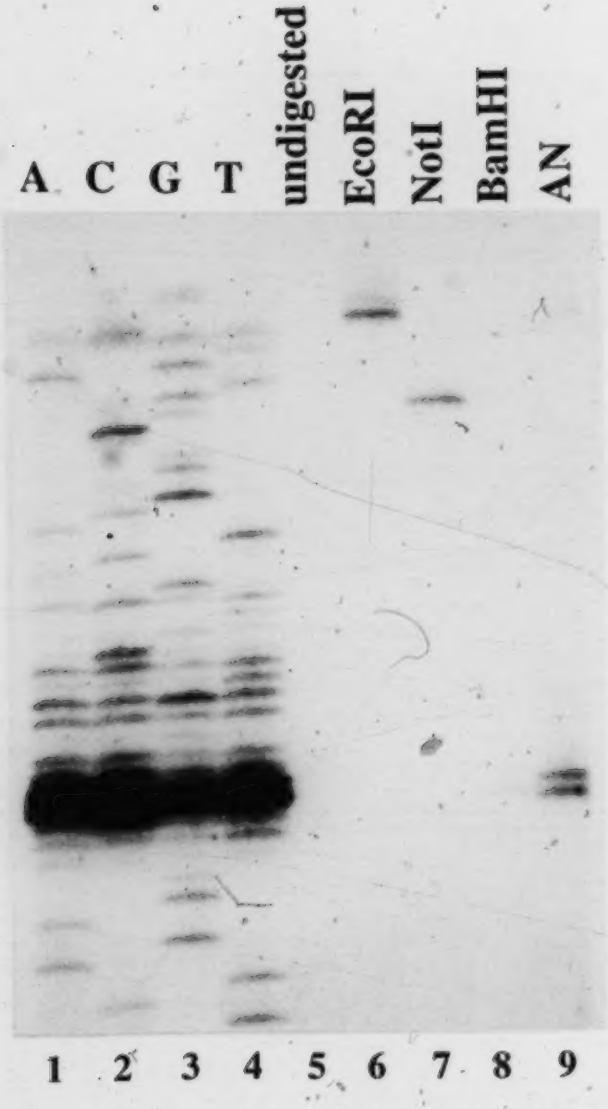

A
C
G
T
undigested
EcoRI
NotI
BamHI
AN
1
2
3
4
5
6
7
8
9

Figure 3.12 Primer extension analysis of pIR using the Rev primer. The products from sequencing and primer extension using labeled Rev primer were analyzed on a 8% polyacrylamide / 7M urea sequencing gel followed by autoradiography. Lanes 1-4 are the sequencing ladder, lanes 5, 6, 7, 8 and 9 are the products from primer extension on undigested pIR, EcoRI, NotI, BamHI, and AN digested pIR, respectively.

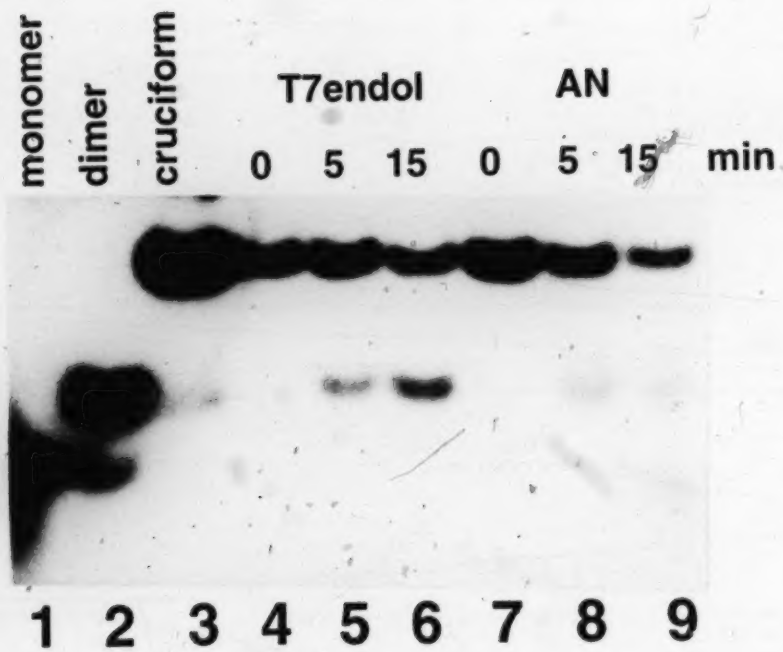

A
C
G
T
undigested
EcoRI
NotI
BamHI
AN
1
2
3
4
5
6
7
8
9

3.3.5 Cleavage of synthetic cruciform

The Holliday resolvases *E. coli* RuvC, T4 endonuclease VII, and T7 endonuclease I have demonstrated the ability to digest synthetic cruciforms created from oligonucleotides to form duplex products (Dickie *et al.*, 1987b; Parsons *et al.*, 1990; Connolly *et al.*, 1991). As well, in chapter 2, the partially purified nuclease was shown to digest synthetic cruciforms. Here, we show that resolvase activity can be demonstrated on synthetic oligonucleotide cruciforms (fig 3.13).

Synthetic cruciforms were labeled at the 3' end by filling in with the Klenow fragment of *E. coli* DNA polymerase I and [α-^{32}P]dCTP and digested with T7 endonuclease I and HSV-1 AN. Digestion of these cruciforms with AN shows the production of products that correspond to the size of a linear duplex formed from annealing only 2 of the oligonucleotides used to make the cruciform (dimer). These same dimer products are also generated from T7 endonuclease I cleavage. Therefore, we can show that digestion of artificial branched DNA by HSV-1 AN can result in the production of linear duplex products, which demonstrates activity consistent with that of a Holliday resolvase.

Figure 3.13 Cleavage of synthetic cruciforms. Cruciforms were constructed from 4 partially complementary oligonucleotides to form a 4 stranded structure that is analogous to a Holliday junction. The cruciform was 3' end labeled with [α^{32}P]dCTP and the Klenow fragment of *E.coli* DNA polymerase I before digestion. Labeled cruciform was digested with either 1U of T7 endonuclease I or 0.0025U of AN for the times as indicated in the figure before termination. For sizing the products, a monomer composed of oligonucleotide M4 alone and a dimer composed of oligonucleotides M1 and M4 were annealed together. The monomer and dimer were labeled with [α^{32}P]ATP and T4 kinase. Samples were analyzed on a 8% nondenaturing polyacrylamide gel followed by autoradiography. Lane 1 is monomer, lane 2 is dimer, lane 3 is undigested cruciform, lanes 4-6 are samples from cruciform digested with T7 endonuclease I, and lanes 7-9 are samples from cruciform digested with AN.


monomer
dimer
cruciform
T7endol
AN
0 5 15 0 5 15 min
1 2 3 4 5 6 7 8 9

3.4 Discussion

In this chapter, we have provided evidence that a Holliday resolvase activity can be demonstrated with purified HSV-1 AN. In previous work presented in chapter 2, a Holliday resolvase activity was found in a crude lysate prepared from HSV-1 infected cells. This has not previously been reported. Furthermore, it was suggested the HSV-1 AN was involved in resolution of cruciforms, but it was not known if AN was responsible for the activities or if the resolving activity was an indirect effect through the interaction with other viral or host proteins. In chapter 2, this could not be determined due to the crude enzyme preparation, which would contain co-purifying proteins of HSV-1 or host cell origin. Thus, it was necessary to obtain highly purified AN. This was achieved using a heterologous expression system rather than AN derived from HSV-1 infection of mammalian cells. The *E. coli* system was chosen because of the ease of obtaining large amounts of purified protein.

We have demonstrated that the purified AN preferentially cleaves cruciform plasmids over other supercoiled plasmids lacking inverted repeats. The range in size of cruciform that was recognized and cleaved by AN was large and there appeared to be no sequence specificity because even a cruciform composed of randomly chosen sequences, as in pIR, or entirely of AT pairs, as in pUC(AT) was efficiently digested. Also, we were able to precisely determine the sites of cleavage by AN. The digestion of pSCB1a by HindIII after AN cleavage produces fragments that were diagnostic of Holliday resolution. Random nicking would have produced only linearization of the plasmid by HindIII. Also, double stranded breaks at other sites would unlikely yield the expected products. We also showed that in pIR most cleavage occurred within the inverted repeat. By fine mapping using the primer extension method, we determined that cleavage of pIR occurred at the base of the cruciform. A resolvase should be able to make symmetrical cuts across the axis of a branchpoint in either direction. This

would result in two cleavage products with either Fwd or Rev primers. Each primer produced two major bands, both corresponding to the restriction enzyme cleaved products at the base of the cruciform formed by the inverted repeat. Finally, we show that AN was able to digest synthetic cruciforms to produce products that were linear duplex in size. Given these results, it was most likely that HSV-1 AN possessed Holliday resolvase activity..

The results presented here are contradictory to those presented by Goldstein and Weller (1998a, 1998b). A mutant that lacked exonuclease activity, but retained endonuclease activity (D340E) was unable to complement AN-1, a null mutant of HSV-1 AN that is defective in egress of capsids from the nucleus of infected cells. Goldstein and Weller (1998a) concluded that the exonuclease activity of AN was required for productive infection. Furthermore, using artificial cruciforms created from oligonucleotides, these authors found that AN completely degraded the structures instead of resolving them into linear duplexes, while D340E appeared to have no activity (Goldstein and Weller, 1998b). This led to the conclusion that AN is not a resolvase. They also showed that AN was active on nicked or gapped DNA found in DNA replication intermediates and suggested that the exonuclease activity was involved in a repair or processing step to remove aberrant secondary structures during replication so that the replicated genome was in a form suitable for packaging.

A possible explanation for the lack of detection of resolvase activity on artificial branched structures by Goldstein and Weller (1998b) was that the cruciforms were 5'-end labeled. The exonuclease activity of HSV-1 AN degrades DNA from 5' to 3' (Knopf and Weisshart, 1990). The free ends of the artificial cruciforms are substrates for AN. Thus, the first product released by a 5'-3' exonuclease from a 5'-end labeled substrate would be the free labeled nucleotide. This was the result seen by Goldstein and Weller (1998b) and taken as evidence of complete degradation. We were able to

see the formation of a linear duplex product because our cruciforms were 3'-end labeled.

Our findings that demonstrate Holliday resolvase activity do not preclude the idea that exonuclease activity is an important function in the processing of DNA. The existence of Holliday resolvase activity fits the model that the branches formed during replication need to be resolved for successful packaging (Shao *et al.*, 1993; Martinez *et al.*, 1996; Severini *et al.*, 1994, 1996). Previous studies on the structure of replicating AN-1 DNA showed that the DNA was in an even more complex form than the wild type DNA (Martinez *et al.*, 1996). Both the resolution of the branches and repair of aberrant structures by endonuclease and exonuclease activities, respectively, may be necessary to properly release a monomer genome from the replicating complex of branched DNA in a form suitable for proper packaging. Although the results that showed a lack of complementation of AN-1 by D340E are compelling (Goldstein and Weller, 1998a), it would be more convincing if a mutant was produced with exonuclease activity but no endonuclease activity that could complement AN-1 mutants. As well, endonuclease was determined by using pUC18 as a substrate whereas Holliday resolvase activity is detected using plasmids with inverted repeats. It is possible that the D340E mutant may have an endonuclease activity but not a resolvase activity. If so, the lack of complementation would be expected. I believe AN has a Holliday resolvase activity. It may be that both activities are necessary for proper packaging and thus egress of capsids from the nucleus.

The results presented in this chapter demonstate that AN has specific cleavage of cruciform plasmids and cleavage occurs at the base of the cruciform. Also, AN can cleave synthetic cruciforms to form linear duplex products. These activities are consistent with AN acting as a Holliday resolvase.

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Chapter 4
Study of Nuclease Activity by Analysis of Mutations Generated in the Herpes Simplex Virus Type 1 Alkaline Nuclease

4.1 Introduction

The UL12 open reading frame of HSV-1 encodes for alkaline nuclease (Costa *et al.*, 1983; Draper *et al.*, 1986; McGeoch *et al.*, 1986, 1988). Homologs of UL12 have been found in other herpesviruses and include herpes simplex virus type 2 (Draper *et al.*, 1986), Epstein-Barr virus (Baer *et al.*, 1984), human cytomegalovirus (Bankier *et al.*, 1991) , pseudorabies virus (DeWind *et al.*, 1994), and bovine herpesvirus type 1 (Vicek *et al.*, 1995). The homologous proteins from these herpesviruses have been isolated and characterized and they have similar endo-exonuclease activities to UL12 of HSV-1 (Hoffmann and Cheng, 1978, 1979; Banks *et al.*, 1983; Baylis *et al.*, 1989, 1991; Stolzenberg and Ooka, 1990; Lin *et al.*, 1995; Hsiang *et al.*, 1996; Chung and Hsu, 1997; Sheaffer *et al.*, 1997). It was found that the human cytomegalovirus (HCMV) counterpart for UL12, called UL98, was able to complement the defects of a null mutant of HSV-1 AN (called HSV-1 UL12lacZ) when supplied in trans or when the UL98 gene was integrated into the HSV-1 UL12lacZ genome (Gao *et al.*, 1998). Thus, there is also a functional conservation of alkaline nucleases between HSV-1 and HCMV. As well, Goldstein and Weller (1998a) have done sequence alignments and have found homologs of the UL12 gene in varicella-zoster virus, human herpesvirus type 6, human herpesvirus type 7, Karposi sarcoma-associated herpesvirus (or HHV-8), equine herpesvirus type 2, murine gammaherpesvirus 68, murine cytomegalovirus and alcelaphine herpes virus type 1.

Figure 4.1 The seven conserved motifs of alkaline nuclease. Seven conserved motifs, labelled I - VII, were identified by sequence alignment of the herpesviruses UL12 homologs in herpes simplex virus type 1, herpes simplex virus type 2, equine herpesvirus type 1, pseudorabies virus, bovine herpesvirus type 1, varicella-zoster virus, herpesvirus saimiri, Karposi's sarcoma-associated virus (human herpesvirus type 8), equine herpesvirus type 2, murine gamma herpesvirus 68, alcelaphine herpes virus type 1, Epstein-Barr virus, human herpesvirus type 6, human herpesvirus type 7, human cytomegalovirus, and murine cytomegalovirus (Goldstein and Weller, 1998a). (A) Map of the location of the conserved motifs in herpes simplex type 1 UL12. (B) Consensus residues of the conserved motifs are shown and their location is indicated above consensus residues. Figure adapted from Goldstein and Weller, 1998a and Martinez *et al.*, 1996.

A.

B.

Motif I 218 VE - - TRGQS - N - LW - LLR - - - - TAS - L 244

Motif II 325 GLL - DP - - G - - GASLD 340

Motif III 362 YEIKCRFKY - F - - - D 377

Motif IV 415 F - - SI - - P - VEY - - - - - - P - - - E - L - T - D - - W 445

Motif V 455 N - - - - S - VM - F 465

Motif VI 491 VF - NPRH - - F - QILVQ - YV - - - YF 514

Motif VII 565 IPV - LIVTPV - V 576

This analysis revealed 7 conserved motifs or domains (motif I to motif VII). The location of the motifs on the UL12 gene and the conserved residues in each motif are illustrated in figure 4.1. The conservation of this protein in herpesviruses suggests that it plays an important and unique role in the life cycle of the virus.

Mutational analysis has been done on HSV-1 AN in order to understand what regions or domains of the protein are required for nuclease activity. Nuclease activity was retained when 126 amino acids from the N-terminus were deleted (Henderson *et al.*, 1998). This is the least conserved region of the herpesvirus ANs. This was supported by a study of Bronstein *et al.* (1997) which demonstrated that a N-terminally truncated version of UL12, designated UL12.5 and encodes residues 127-626, retained the same activity as wild type. In contrast, the C-terminal region is highly conserved and was important for nuclease function. The 7 conserved motifs all fall downstream of residue 127. As few as 49 amino acids deleted from the C-terminus completely abrogated nuclease activity (Henderson *et al.*, 1998).

Two mutants were generated in one of the most highly conserved of the 7 motifs called motif II (amino acid residues 325 to 340) (Goldstein and Weller, 1998a). The high conservation suggests that these regions are important for nuclease activity. The aspartic acid at residue 340 was changed to a glutamic acid and designated D340E. The other mutant was a two amino acid substitution in which glycine at residue 336 and serine at 338 were changed to alanine. This mutant was designated as G336A/S338A. Mutant G336A/S338A had no activity while D340E had no exonuclease activity but retained endonuclease activity, as measured by digestion of pUC18 (Goldstein and Weller, 1998a).

We wanted to extend the number of mutations in UL12, particularly within the conserved motifs to investigate the contribution of other residues to enzyme activity,

measured as described in the preceding chapters. In nucleases that have a divalent metal ion requirement, acidic residues are important for metal binding and thus catalysis (Suck, 1992; Gerit, 1993). The acidic residues bind metal cations to the active site. These metal cations can act as a proton acceptor for water to create a hydroxide nucleophile for phosphodiester bond cleavage. Acidic residues have been implicated as important for activity in the resolvases *E. coli* RuvC, T4 endonuclease VII, and T7 endonuclease I (Saito *et al.*, 1995; Giraud-Panis, 1996; Parkinson *et al.*, 1999) Therefore, we targeted the conserved acidic residues in 4 of the 7 conserved domains (see table 4.1). We made point mutations by *in vitro* mutagenesis at the aspartic acids 329 and 340 (D329 and D340). We substituted aspartic acid with alanine, asparagine, and glutamic acid. Also, mutations were made in glutamic acids 219, 364 and 423 (E219, E364 and E423) by substituting glutamic acid with alanine, glutamine, and aspartic acid. Each of these mutants were examined for exonuclease and endonuclease activities.

4.2 Materials and Methods

4.2.1 Production of mutant alkaline nucleases

Point mutations in AN were constructed using *in vitro* mutagenesis with mutagenic oligonucleotides as shown in table 4.1. The AN gene (UL12) was cloned into pALTER-1 and named pALAN-1 as described in chapter 3 and mutants were generated with the Altered Sites *in vitro* mutagenesis system (Promega, Madison, WI, USA). Briefly, single stranded DNA was produced by transforming pALAN-1 into the *E. coli* strain JM103 which was infected with helper phage M13KO7. The single stranded DNA was isolated and the desired mutagenic oligonucleotide (table 4.1) was annealed to the DNA. The mutant strand was synthesized by extension with T4 DNA polymerase and ligation with T4 DNA ligase (Promega). The DNA was transformed into *E. coli* strain BMH 71-18 mut S, which is a repair minus strain in which repair of the newly synthesized unmethylated strand does not occur. The mutagenized DNA was isolated and transformed into DH5α and the mutations were confirmed by sequencing with the double-stranded DNA cycle sequencing kit (Gibco BRL, Gaithersburg, MD, USA). The mutated sequences were digested with EcoRI and HindIII and subcloned into the EcoRI-HindIII sites of pRSETB (Invitrogen, Carlsbad, CA, USA) for expression in *E. coli*.

4.2.2 Expression and purification

The subcloned mutant nucleases were transformed into the *E. coli* strain MC1061(pT7pol26) cells for expression (Mertens *et al.*, 1995). The cells were grown in LB medium supplemented with 100μg/mL ampicillin, 25μg/mL kanamycin and 10mM glucose at 37°C to an OD_{600} of 0.6 - 0.7. The production of mutant nuclease was induced by adding IPTG to a final concentration of 1mM and incubation was continued at 37°C for another 3 hrs.

The induced cells were harvested by centrifugation, washed once in 1/10 culture volume with 10mM Tris-HCl pH 8, 0.1mM EDTA, and centrifuged again. Cells were resuspended in 1/10 culture volume with TSE (20mM Tris-HC l pH 8.0, 0.1M NaCl, 10mM EDTA) with 0.1mM PMSF added. Lysozyme was added to 1mg/mL and the cells were incubated at room temperature for 10 min with continuous mixing. The cell suspension was frozen to -70°C and quickly thawed at 37°C. The suspension was sonicated and insoluble material was pelleted at 10,000 x g for 10 minutes. Most of the mutant AN was found in the insoluble fraction in the inclusion bodies (fig. 4.2). The insoluble pellet was washed twice with 1/10 culture volume with TSE and the remaining insoluble material was pelleted by centrifugation. The insoluble pellet was solubilized in a small volume of 1%SDS, 10mM DTT, 20mM Tris-HCl pH 8, 0.1mM EDTA, 0.1mM PMSF at room temperature. The solubilized inclusion bodies were quickly diluted with 20mM Tris-HCl pH 8, 0.1mM EDTA, 0.5M NaCl, 1mM DTT, 0.1mM PMSF, 2µg/ml aprotinin, 1µg/mL leupeptin, 1µg/mL pepstatin, and 20% glycerol so that the concentration of SDS was 0.025%. Triton X-100 was added to a final concentration of 1%, incubated for 5 min at room temperature and spun down at 10,000 x g for 10 min.

Since expression results in mutant nuclease with a N-terminal 6xHis tag, purification on Ni-NTA resin (Qiagen, Hilden, Germany) was performed. All purification steps were carried out at 4°C. Solubilized mutant nuclease extract was bound to Ni-NTA for 1hr. The resin was washed twice with 10 column volumes with 20mM Tris-HCl pH 8, 0.3M NaCl, 10mM imidazole, 0.1mM PMSF, 5mM β–mercaptoethanol. Elution was performed 4 times with 1 column volume fractions with 20mM Tris-HCl pH 8, 0.15M NaCl, 250mM imidazole, 10mM β-mercaptoethanol, 0.1mM PMSF, 2µg/mL aprotinin, 1µg/mL leupeptin, 1µg/mL pepstatin, 0.1mM EDTA, 20% glycerol. Eluted nuclease was pooled and dialyzed

overnight at 4°C against 20mM Tris-HCl pH 8, 0.1mM EDTA, 1mM DTT, 0.1mM PMSF, 20% glycerol. Dialyzed nuclease was centrifuged to remove any precipitated material and the purified nuclease was stored at -20°C. Protein concentrations were determined using the Bio-Rad (Hercules, CA, USA) DC protein assay.

4.2.3 Detection of nuclease activity

Exonuclease and endonuclease activity were initially determined using an ethidium bromide based fluorescence assay as described in chapter 2 (Morgan *et al.*, 1979a, 1979b). Intercalation of ethidium bromide into double stranded DNA will give an intensity of fluorescence when measured on a fluorometer which gives a relative concentration of the amount of double stranded DNA present in a sample. Degradation of DNA was observed as a decrease in the intensity of fluorescence. Nuclease reactions were performed in a reaction buffer containing 50mM Tris-HCl (pH 7.5 or 9), 10mM $MgCl_2$ or $MnCl_2$, 1mM DTT, 100µg/mL BSA, and the appropriate DNA substrate in a final volume of 100µl. The reaction mixture was kept on ice. The reaction was initiated by adding enzyme to the reaction mixture followed by incubation at 37°C. At various time points, 20µl aliquots were removed and terminated by addition directly to 1.2mL of 20mM K_3HPO_4 pH 11.8, 0.5mM EDTA, 0.5µg/mL ethidium bromide (Morgan *et al.*, 1979a). Fluorescence was measured on a Sequoia - Turner (Mountain View, CA, USA) model 450 digital fluorometer with a NB520 excitation filter and SC605 emission filter.

For exonuclease activity measurements, 2.5µg of sonicated calf thymus DNA (Sigma, St. Louis, MO, USA) in pH 9 buffer with $MgCl_2$ as the cation was used. For endonuclease, 2.5µg of plasmid pSCB1a (DeLange *et al.*, 1986; Stuart *et al.*, 1992) in pH 7.5 buffer with $MnCl_2$ as the cation was used. For endonuclease activity, the sample was denatured for 2 minutes at 95°C for 2 minutes and then quickly cooled to

room temperature in a water bath prior to determination of fluorescence. The assays for activity were done in parallel with the same concentration of purified wild type AN and extract prepared from cells containing only pRSETB (vector only) (Table 4.2)

A more sensitive exonuclease assay was used to measure the release of acid-soluble nucleotides from the digestion of ^{3}H-labeled *E. coli* DNA (NEN Life Science Products, Boston, MA, USA). The labeled DNA was made single-stranded by boiling for 10 minutes and quickly cooled on ice. The reaction mixture contained 50mM Tris-HCl pH 9, 10mM $MgCl_2$, 1mM DTT, 100µg/mL BSA, 25nCi of denatured ^{3}H-labeled *E. coli* DNA and 0.5µg of enzyme in a total volume of 100µl. A reaction containing no enzyme was performed in parallel as a control The reaction mixture was incubated at 37^{o}C for 30 minutes and terminated by the addition of 1 mL of ice cold 5% Trichloroacetic acid (TCA) and 0.25 mg/mL BSA, incubated on ice for 10 minutes, and centrifuged for 5 minutes at 15,000 X g. A 200 µl aliquot of the soluble fraction was added to 7 mL of Ecolite (ICN Biomedicals, Costa Mesa, CA, USA) scintillation fluid and counted in a Beckman (Fullerton, CA, USA) model 6000TA scintillation counter.

4.2.4 Source of other enzymes

The restriction enzymes EcoRI, NotI, and BamHI and the DNA modifying enzymes T4 polynucleotide kinase and Taq DNA poymerase were purchased from Gibco BRL. T7 endonuclease I was purchased from New England Biolabs (Beverly, MA, USA).

4.2.5 Cleavage of cruciform plasmids

The plasmids pSCB1a and pIR contain inverted repeats that, due to the torsion of supercoiling, extrude the repeats into the arms of a cruciform structure. The inverted repeat in pSCB1a is a 1.5kb inverted repeat from the Shope fibroma Virus (DeLange *et*

al., 1986; Stuart *et al.*, 1992). The inverted repeat in pIR was made from annealing two oligonucleotides that have a 44bp inverted repeat flanked by an EcoRI site at one end and a BamHI site at the other. At the axis of the inverted repeat is a NotI site (see Chapter 3, fig 3.11).

Digestion of plasmids was performed in a 100µl of reaction mixture (50mM Tris-HCl pH 7.5, 10mM MgCl2, 1mMDTT, 100µg/ml gelatin and 1µg of pSCB1a or pIR) which was kept chilled on ice until the reactions were initiated by the addition of 0.6µg of nuclease and transferring to 37°C. At the times indicated in the figures, 20µl aliquots were removed and terminated by the addition of 4µl of 0.15M EDTA, 3%SDS. The samples were run on 0.8% agarose/ 0.5XTBE (1X=89mM Tris borate, 2mM EDTA) gels and visualized by staining with ethidium bromide.

4.2.6 Determination of the site of cleavage

The cleavage sites on pIR were mapped by primer extension using the oligonucleotide primer Fwd (5'-CGGGCCTCTTCGCTATTACG) which flanks the inverted repeat (see chapter 3, fig. 3.11). The primer was 5' end labeled with [γ-^{32}P]ATP (NEN Life Science Products) and T4 polynucleotide kinase (Sambrook *et al.*, 1989). Extension of the primer on pIR digested with nuclease was performed with Taq DNA polymerase using initial denaturation conditions of 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 60°C for thirty seconds, 72°C for 90 seconds, and ended with a 5 minute extension at 72°C in a MJ Research PTC-100 thermal cycler (Watertown, MA, USA). The samples were analyzed on standard 8% polyacylamide/7M urea sequencing gels (Sambrook *et al.*, 1989) followed by autoradiography. For comparison, the cleavage sites from undigested pIR, EcoRI, NotI, BamHI, T7 endonuclease I, and wild type AN digested pIR were analyzed by the same method. The sequence of pIR was analyzed using the same primer used for the primer extension using a double stranded

DNA cycle sequencing kit (Gibco BRL).

4.3 Results

4.3.1 Construction of mutants of alkaline nuclease

To investigate the role of selected amino acids required for nuclease activity, point mutations within the AN gene UL12 were constructed. Previous studies have shown that the N-terminal 126 amino acids were dispensable for nuclease activity but a truncation of as few as 49 amino acids from the C-terminal end completely abrogated enzyme activity (Bronstein *et al.*, 1996; Henderson *et al.*, 1998). Seven conserved regions or motifs have been described and these all map downstream of the N-terminal 126 amino acids (Goldstein and Weller, 1998a). As well, 2 mutations in the most highly conserved region (called motif II, fig 4.1) that encompass amino acid residues 325 to 340 were constructed. Both mutants lacked exonuclease activity, but the mutant in which asp340 was substituted by glutamic acid (D340E) retained endonuclease activity as measured by linearization of pUC18 (Goldstein and Weller, 1998a).

In nucleases, acidic residues essential to catalyze the hydrolysis of the phosphodiester backbone of DNA (Suck, 1992; Gerit, 1993) . The acidic residues are important for binding the metal ions required for catalysis. Acidic residues in the resolvases *E. coli* RuvC, T4 endonuclease VII and T7 endonuclease I have been identified as essential for catalysis (Siato *et al.*, 1995; Giraud-Panis and Lilley, 1996; Pohler *et al.*, 1996; Parkinson *et al.*, 1999; White *et al.*, 1997). These acidic residues were identified by site-directed mutagenesis and found to be essential for cleavage but not for junction binding. Considering these previous observations, we targeted conserved acidic residues in the conserved motifs in AN to see if there was an effect on activity.

Point mutations were constructed in aspartic acid residues 329 and 340 and in glutamic acid residues 219, 364 and 423. Three mutations were generated in each of

Table 4.1
List of oligonucleotides used to generate point mutations in HSV-1 alkaline nuclease

Mutant	*Oligonucleotide* [a]
wild type	ATG GGG TTT TAC GAG GCG GCC ACG CAA AAC CA
E219A	ATG GGG TTT TAC G**C**G GC**C** GCC ACG CAA AAC CA
E219Q	ATG GGG TTT TAC **C**A**A** GCG GCC ACG CAA AAC CA
E219D	ATG GGG TTT TAC GA**T** GCG GCC ACG CA
wild type	GT GGG GTC CTC ATG GAC GGT CAC ACG GGG G
D329A	GT GGG GTC CTC ATG G**C**C GGT CAC ACG GGG G
D329N	GG GTC CTC ATG **A**AC GGT CAC ACG GG
D329E	GTC CTC ATG GA**A** GGT CAC ACG GG
wild type	G GCG TCC CTG GAT ATT CTC GTC TGT C
D340A	G GCG TCC CTG G**C**T ATT CTC GTC TGT
D340N	G GCG TCC CTG **A**AT ATT CTC GTC
D340E	GCG TCC CTG GA**G** ATT CTC GTC TGT C
wild type	A GCC TTT TAC GAG GTC AAA TGC C
E364A	GCG TTT TAC G**C**G GTC AAA TG
E364Q	GCC TTT TAC **C**AG GTC AAA TG
E364D	A GCC TTT TAC GA**C** GTC AAA TGC C
wild type	CC GGC CCG GAG GAG GCT CTC GTC ACA C
E423A	CC GGC CCG GAG G**C**G GCT CTC GTC ACA
E423Q	CC GGC CCG GAG **C**AG GCT CTC GTC ACA C
E423D	GGC CCG GAG GA**T** GCT CTC GTC ACA

[a] Oligonucleotides used to make mutants are compared to wild type sequence. Changes from wild type are indicated in bold and underlined.

Figure 4.2 Expression of mutant nucleases. Samples of uninduced cells (lanes 1,6,10), IPTG induced cells (lanes 2,7,11), soluble fraction (lanes 3,8,12), and insoluble fraction (lanes 4,9,13) from expression of mutant nucleases D329A, D329N, and D329E in *E. coli* was screened on a 7.5% SDS polyacylamide gel followed by staining with Coomassie brilliant blue. Size of molecular weight markers (shown in lane 5) is indicated on left of gel (in kilodaltons). The expression of the D329 mutants is representaive of the expression level of all mutants produced.

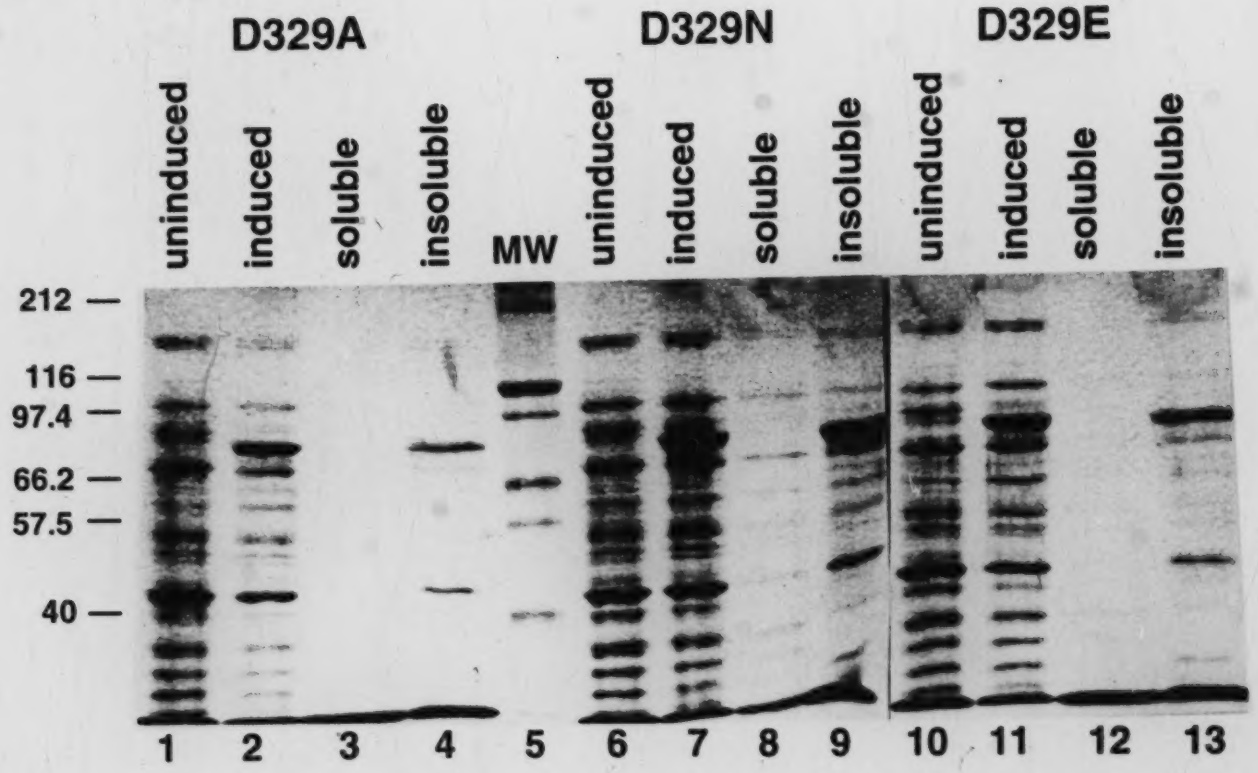
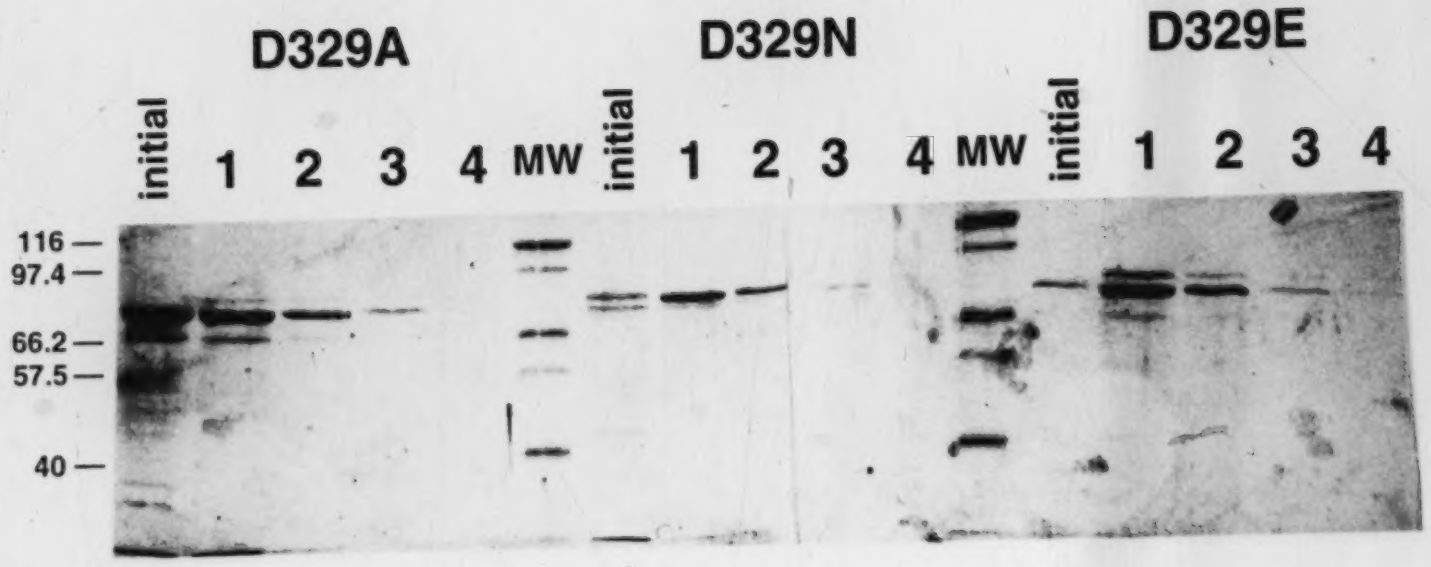

D329A
D329N
D329E
uninduced
induced
soluble
insoluble
MW
uninduced
induced
soluble
insoluble
uninduced
induced
soluble
insoluble
212
116
97.4
66.2
57.5
40
1
2
3
4
5
6
7
8
9
10
11
12
13

Figure 4.3 Purification of mutant nucleases. The insoluble fraction from expression of mutant nucleases was solubilized with SDS and exchanged with Triton X-100 as described in materials and methods and purified by metal affinity chromatography on Ni-NTA agarose was analyzed on a 7.5% SDS polyacylamide gel and stained with Coomassie brilliant blue. A sample of the initial amount of nuclease loaded onto Ni-NTA agarose is shown and the numbers indicate the fraction collected from elution. The size of the molecular weight markers (in kilodaltons) are shown on the left and seen in lanes designated MW.


D329A
D329N
D329E
initial
1
2
3
4
MW
initial
1
2
3
4
MW
initial
1
2
3
4
116
97.4
66.2
57.5
40

the residues chosen for analysis (table 4.1). All residues had a substitution of the acidic residue to alanine and were called D329A, D340A, E219A, E364A, and E423A. The second type of mutation were changes to residues that had the same side chain length as the acidic residues but had an amine instead of the carboxy group (asparagine and glutamine). These mutants were designated as D329N, D340N, E219Q, E364Q, and E423Q. The final set of mutations were conservative mutations in which aspartic acids and glutamic acids were changed to glutamic acids and aspartic acids, respectively, and these were designated D329E, D340E, E219D, E364D, and E423D.

4.3.2 Expression and purification of mutant alkaline nucleases

The mutant ANs were expressed in a T7 promoter based *E. coli* expression system as was previously described with wild type alkaline nuclease (chapter 3). As with wild type, expression of the mutant ANs resulted in the production of insoluble protein (fig 4.2, lanes 4, 9, 13). The inclusion bodies were solubilized with SDS and exchanged with Triton X-100 before purification by metal affinity chromatography on NI-NTA agarose (fig 4.3). Expression and purification yielded protein of approximately 87kDa, which was the same size as wild type AN, indicating that the expressed mutant proteins appeared to be full length.

4.3.3 Nuclease activity of mutant alkaline nucleases

Exonuclease and endonuclease activity of each mutant nuclease was determined using a fluorescence based assay (Morgan *et al.*, 1979a, 1979b). Intercalation of ethidium bromide into double stranded DNA results in fluorescence that is relative to the amount of double stranded DNA in a sample. Digestion of DNA would therefore be seen as a drop in fluorescence.

Table 4.2

Nuclease Activity of Mutants of Alkaline Nuclease

samples [a]	*Exonuclease* [b]				*Endonuclease* [c]			
	0min[d]	60min[e]	Δ F[f]	%WT[g]	0min[d]	60min[e]	Δ F[f]	%WT[g]
pRSETB	382	370	12	4.1	170	155	15	14.9
wild-type	294	0	294	100	206	105	101	100
E219A	270	275	-5	-1.7	156	151	5	5.0
E219Q	280	300	-20	-6.8	155	168	-13	-12.9
E219D	304	338	-34	-11.6	143	144	-1	-1.0
D329A	248	272	-24	-8.2	122	52	70	69.3
D329N	246	266	-20	-6.8	126	57	69	54.8
D329E	307	330	-23	-7.8	131	66	65	64.4
D340A	332	339	-7	-2.4	158	140	18	17.8
D340N	338	354	-16	-5.4	157	152	5	5.0
D340E	341	329	12	4.1	130	131	-1	-1.0
E364A	240	244	-4	-1.4	170	170	0	0
E364Q	214	234	-20	-6.8	157	166	-9	8.9
E364D	266	247	19	6.5	147	147	0	0
E423A	317	3	314	106.8	152	77	75	74.3
E423Q	323	12	311	105.8	153	73	80	79.2
E423A	335	2	233	113.3	152	51	101	100

[a] pRSETB (vector only), wild-type AN, and mutant nucleases expressed in *E. coli*

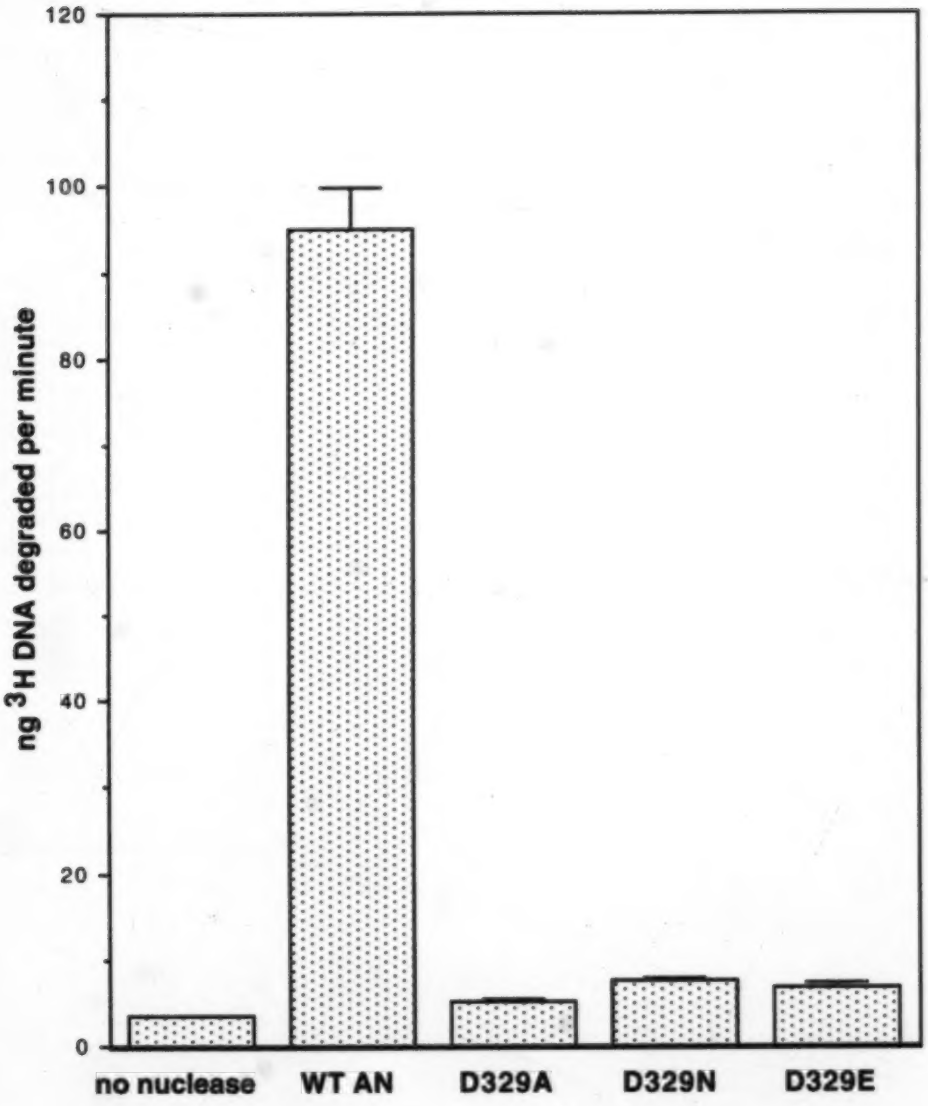
[b] Exonuclease activity determined by fluorescence assay at pH 9 with $MgCl_2$

[c] Endonuclease activity determined by fluorescence assay at pH 7.5 with $MnCl_2$

[d] Initial fluorescence in sample [e] Fluorescence in sample after 60 minutes of degradation

[f] change in fluorescence from initial time to 60 minutes [g] percentage of nuclease activity compared to wild-type where wild-type level is 100%

Figure 4.4 Exonuclease activity of D329 mutants. The purified enzyme expressed from wild type alkaline nuclease (WT AN) and mutants D329A, D329N and D329E or no nuclease were incubated with ^{3}H-labelled DNA as described in materials and methods. Exonuclease was measured as the release of acid-soluble radiolabelled nucleotides in nanograms per minute. The mean and standard deviation from 3 independent samples are plotted.


120
100
80
60
40
20
0
ng ^{3}H DNA degraded per minute
no nuclease
WT AN
D329A
D329N
D329E

To test for exonuclease activity, calf thymus DNA was used as substrate. The reactions were performed at pH 9 and with $MgCl_2$ because these conditions favor alkaline exonuclease activity. For endonuclease activity, the ccc plasmid pSCB1a wasused. The reaction was performed with $MnCl_2$ to inhibit exonuclease activity. Also, to detect endonuclease activity, the sample was denatured and quickly cooled before the fluorescence was measured. If the plasmid was nicked by an endonuclease activity, the 2 strands of DNA would not be able to anneal with the quick cooling and this would be detected as a drop in fluorescence. These assays were done in parallel with wild type AN and extract produced from vector only (pRSETB) for comparison. As summarized in table 4.2, only the AN with mutations in D329 and E423 had any activity. Mutations in D340, E364, and E219 lacked detectable activity above the pRSETB control, indicating that these residues are essential for activity. Mutations in E423 do not seem to affect activity to a large extent, indicating that this residue is unlikely needed for catalysis. Exonuclease activity is at wild-type levels in all the E423 mutants and endonuclease activity is only slightly reduced compared to wild-type AN in E423A and E423Q. Interestingly, all the mutations in D329 cause loss of exonuclease activity, but there was retention of endonuclease activity. It has been reported that the mutant D340E also had no exonuclease activity and retained endonuclease activity (Goldstein and Weller, 1998a). We were unable to detect this phenotype possibly because the fluorescence assay is not sensitive enough. The endonuclease activity of D340E was reported to be somewhat reduced compared to wild type (Goldstein and Weller, 1998a). Also, as previously seen in chapter 3, the specific activity of wild type nuclease is not as high when compared to published reports (Bronstein and Weber, 1996) because we use $MnCl_2$ for our assays. As well, the pH of the reaction buffer used in the fluorescence assay was pH 7.5, which is sub-

optimal for an alkaline nuclease (Hoffmann and Cheng, 1978, 1979; Strobel-fidler and Francke, 1980; Banks *et al.*, 1983, Bronstein and Weber, 1996). The reaction conditions used in this thesis were optimized for detection of Holliday resolvase activity, whereas detection of endonuclease activity in D340E by Goldstein and Weller (1998a) was based on digestion of pUC18. Since D329 mutants were able to demonstrate endonuclease activity in the absence of exonuclease activity, we used these mutants only in the remaining experiments for characterization.

To confirm that the mutants in D329 were deficient in exonuclease activity, a more sensitive assay based on the release of acid-soluble nucleotides from ^{3}H-labeled *E. coli* DNA was used. As illustrated in fig 4.4, the level of exonuclease was markedly reduced compared to wild type exonuclease and was close to background levels.

4.3.4 Cleavage of plasmid cruciforms

Evidence for Holliday resolvase activity has been presented for wild type AN (chapter 3). Therefore, we wanted to see if mutants lacking exonuclease but that had retained endonuclease activity could have Holliday resolvase activity. First, we checked for cleavage of plasmid cruciforms pSCB1a (fig 4.5) and pIR (fig 4.6). Cleavage of pSCB1a by the nucleases resulted in the production of a linear product (fig 4.5). In wild type, the product disappeared over time presumably due to exonuclease activity. In contrast, in the reaction mixtures containing D329 mutants, the product accumulated because the linear product was not digested by exonuclease activity. A similar result was observed when pIR was the substrate (fig 4.6). The wild type AN digestion resulted in loss of product due to exonuclease but this loss of product was not seen with the D329 mutants.

Figure 4.5 Cleavage of cruciform plasmid pSCB1a by wild type and mutant alkaline nucleases. The products from digestion of pSCB1a by wild type AN (lanes 4-7), D329A (lanes 8-11), D329N (lanes 12-15), and D329E (lanes 16-19) for the time (in minutes) indicated at the top of the lanes were analyzed on a 0.8% agarose/0.5XTBE gel and stained with ethidium bromide. Lane 1 is size marker of λ DNA digested with HindIII, lane 2 is undigested pSCB1a and lane 3 is EcoRI digested pSCB1a. The position and size of linearized pSCB1a is indicated by the arrow on the right.

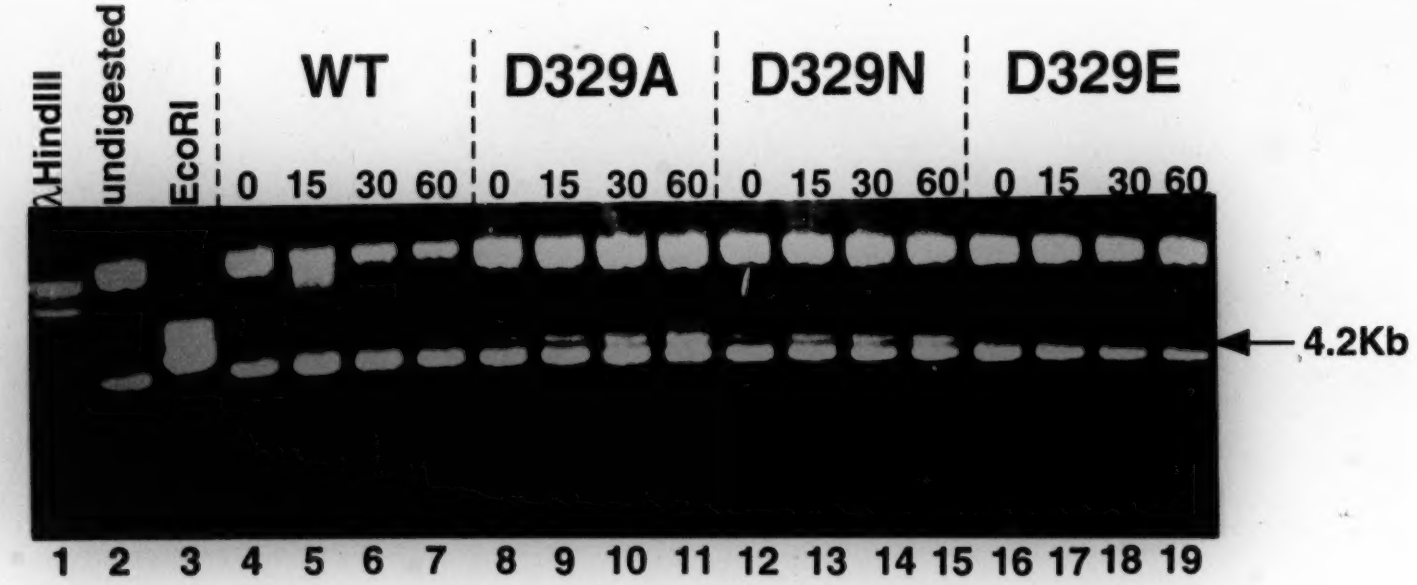

λHindIII
undigested
EcoRI
WT
D329A
D329N
D329E
0 15 30 60 0 15 30 60 0 15 30 60 0 15 30 60
4.2Kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 4.6 Cleavage of cruciform plasmid pIR by wild type and mutant alkaline nucleases. Products from digestion of pIR with wild type AN (lanes 2-5), D329A (lanes 6-9), D329N (lanes 10-13), and D329E (lanes 14-17) for the times (in minutes) indicated at the top of the lanes were analyzed on a 0.8% agarose/0.5XTBE gel and stained with ethidium bromide. Lane 1 is size marker of λ DNA digested with HindIII. The position of the open circular (oc), linear, and supercoiled (ccc) forms of pIR are indicated on the right.

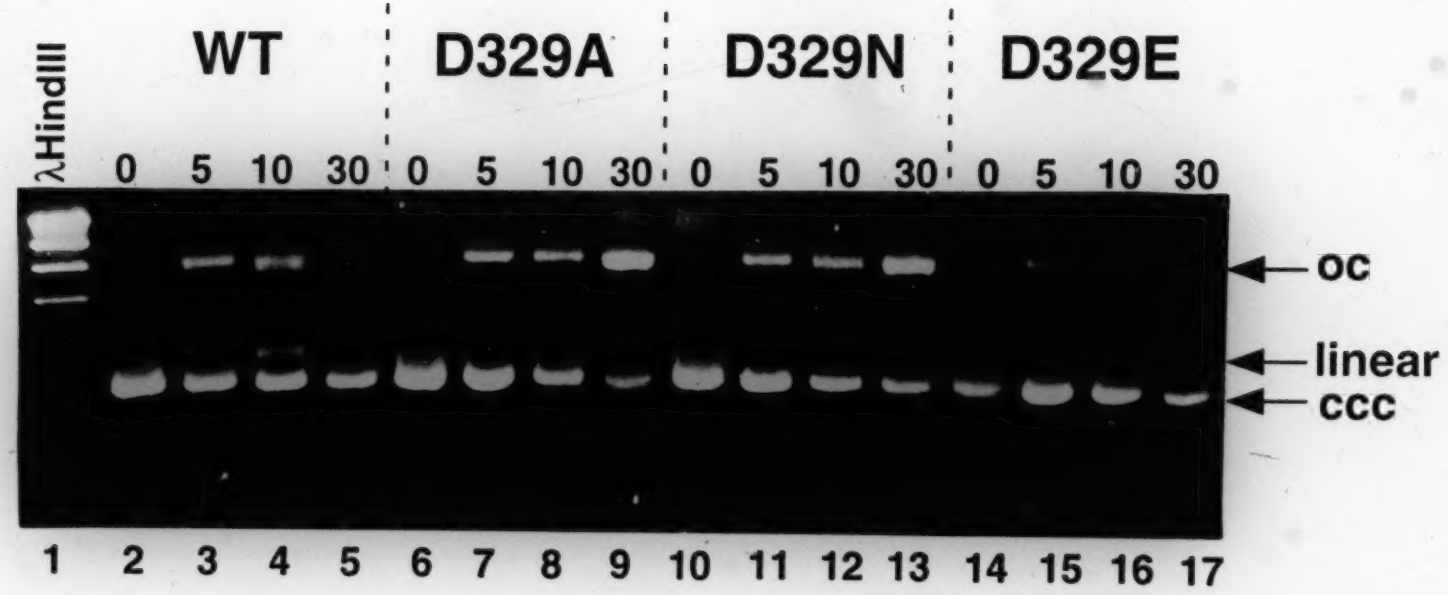

λHindIII
WT
D329A
D329N
D329E
0 5 10 30 0 5 10 30 0 5 10 30 0 5 10 30
oc
linear
ccc
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 4.7 Mapping the cleavage sites of pIR by mutant nucleases. Primer extension was performed on digested pIR using the Fwd primer as described in chapter 3 and in materials and methods. Lanes 1-4 show sequencing ladder around the inverted repeat of pIR. The products from primer extension from undigested pIR (lane 5) and cleavage by EcoRI (lane 6), NotI (lane 7), BamHI (lane 8), T7 endonuclease I (lane 9), Wild type AN (lane 10), D329A (lane 11), D329N (lane 12), and D329E (lane 13) were analyzed on a 8% polyacrylamide / 7M urea sequencing gel followed by autoradiography.

A C G T
1 2 3 4 5 6 7 8 9 10 11 12 13
undigested
EcoRI
NotI
BamHI
T7 endo I
ΔN
D329A
D329N
D329E

4.3.5 Mapping of the cleavage sites

The sites of digestion by the D329 mutants were mapped by a primer extension method using the Fwd primer for pIR (chapter 3 , fig 3.11). The products from digestion by the D329 mutants were compared to those generated by EcoRI, NotI, BamHI, T7 endonuclease I, and wild type AN digestion of pIR. The Fwd primer was also used to sequence undigested pIR. As seen in fig 4.7, the major products from D329 mutant cleavage map to the EcoRI and BamHI sites, which are at the branchpoint at the base the cruciform in pIR. These products are also the same formed by T7 endonuclease I and wild type AN cleavage. Therefore, it appears that the D329 mutants retained Holliday resolvase activity in the absence of exonuclease activity.

4.4 Discussion

Five residues that are conserved among herpesviruses were mutated in order to assess the importance of these residues and their requirement in AN activity. The residues chosen were acidic residues, which are known to be important for activity in other nucleases. These residues usually bind metal cations needed for catalytic activity (Suck, 1992; Gerit, 1993). In the Holliday resolvases *E. coli* RuvC, T4 endonuclease VII and T7 endonuclease I, it has been found that acidic residues were important for catalytic activity but unnecessary for junction binding (Saito *et al.*, 1995; Giraud-Panis and Lilley, 1996; Pohler *et al.*, 1996; Parkinson *et al.*, 1999; White *et al.*, 1997). The residues chosen were E219 (in motif I; amino acids 218 to 244), E364 (in motif III; amino acids 362 to 377), D329 and D340 (in motif II; amino acids 325 to 340), and E423 (motif IV; amino acids 415 to 445). The mutations in aspartic acids consisted of changes to alanine, asparagine and glutamic acid. Mutations in glutamic acids consisted of changes to alanine, glutamine or aspartic acid. It was found that all the mutations made in E219, E364, and D340 resulted in loss of endonuclease and exonuclease activities as determined by the ethidium bromide fluorescence assay. The D340E mutant was generated previously (Goldstein and Weller, 1998a) and was described as having no exonuclease activity, but retained endonuclease activity. I did not detect any endonuclease in this mutant in our assay. This is presumably due to the assay conditions in which $MnCl_2$ was used in a reaction buffer at pH 7.5. These reaction conditions were not optimal for activity when tested on a supercoiled plasmid (Hoffmann and Cheng, 1978, 1979, Strobel-fidler and Francke, 1980; Banks *et al.*, 1983; Bronstein and Weber, 1996). These conditions were optomized for Holliday resolvase activity.

The mutations at D329, however, produced a series of mutants that were deficient in exonuclease but retained endonuclease activity. This was the second set of mutants in motif II that resulted in this phenotype (see Goldstein and Weller, 1998a). The mutants at D329 were interesting in that they showed that the exonuclease and endonuclease activities were separable. However, we did not find a mutant that had retained exonuclease activity but lost endonuclease activity.

We provided evidence that HSV-1 AN had a Holliday resolvase activity (chapter 3). We wanted to see if the D329 mutants had resolvase activity which would be expected if Holliday resolvase was a real activity of AN. We found that under reaction conditions using $MgCl_2$, which favors exonuclease activity, a linear product from plasmid digestion accumulated, whereas with wild type AN, the product disappeared, presumably due to exonuclease activity. The plasmids used were pSCB1a (DeLange *et al.*, 1986; Stuart *et al.*, 1992) and pIR (chapter 3). These plasmids contain inverted repeats of 1.5kb and 44bp, respectively. Upon supercoling, the inverted repeats can extrude into cruciforms, which are analogs of Holliday junctions (Dickie *et al.*, 1987a).

We mapped the sites of digestion with the primer extension method and found that the digestion sites mapped to restriction enzyme sites at the base of the cruciform. The cleavage sites in the product digested by the mutants D329A, D329N, and D329E were the same as the digestion products from wild type AN and T7 endonuclease I, a known resolvase (de Massey *et al.*, 1987; Dickie *et al.*, 1987b, Picksley *et al.*, 1990). Thus, it appeared that endonuclease activity of AN was also responsible for the Holliday resolvase activity.

The mutant D340E, was previously reported to have lost exonuclease activity, but retained endonuclease activity (Goldstein and Weller, 1998a). However, the D340E that I expressed and purified lacked detectable activity. One possibility for lack

of detection is that the protein is improperly folded. D340E was expressed in baculovirus by Goldstein and Weller (1998a) and is soluble in this expression system. *E. coli* expression resulted in the formation of insoluble inclusion bodies that had to be denatured and refolded. Another possibility may be that D340E may be able to digest a supercoiled plasmid like pUC18, but the mutations causes loss of Holliday resolvase activity. The assay conditions used to initially screen for activity (table 4.2) used a cruciform plasmid in a pH 7.5 buffer with $MnCl_2$ as the cation. Under these conditions, we were able to easily detect Holliday resolvase activity. It is less likely that D340E was misfolded because wild-type AN, E423 mutants, and D329 mutants are refolded under the same conditions and they display activity, but these activities were evaluated under conditions that favor detection of Holliday resolvase activity.

We have created mutants in AN residues E219, E364 and D340 that have no nuclease activity. We speculated that these acidic residues were important for catalytic activity. However, it was possible that these sites may have been important for binding. We did not do any binding studies but, presumably, a defect in binding would cause loss of activity. It is currently not known what residues are important for binding of AN to DNA. In *E. coli* RuvC, crystallographic analysis of the protein has lead to the identification of residues, mainly basic residues such as lysine and arginine, that may be involved in DNA binding (Aryoshi *et al.*, 1994). In T7 endonuclease I, lysine and arginine residues were shown to be involved in DNA junction binding (Parkinson *et al.*, 1999). In pseudorabies virus (PRV), a DNA binding domain has been identified in the AN homolog that is located at residues 274-492 (Ho *et al.*, 2000). It was shown that a mutant that truncated the first 274 amino acids had a 7 fold increase in DNA binding ability.

Another consideration is the contribution of other residues to nuclease activity that are not acidic amino acids. In HSV-1, the mutant G336A/S338A in motif II caused

loss of endonuclease and exonuclease activity (Goldstein and Weller, 1998a). In Epstein-Barr virus UL12 homolog, mutation of leucine 23 to glycine , which corresponds to HSV-1 UL12 leucine 150, completely abolished enzyme activity (Lin *et al.*, 1994). A mutation of leucine 150 of HSV-1 to lysine also abolished nuclease activity but this amino acid was not in any of the conserved motifs (Goldstein and Weller, 1998a). In PRV AN, a histidine residue at amino acid 371, which corresponds to amino acid 497 in HSV-1 UL12, which is in motif VI (Goldstein and Weller, 1998a), was mutated to alanine and displayed a loss of endo-exonuclease activity, but retained DNA binding ability (Ho *et al.*, 2000).

In this chapter, we have identified several new mutations in AN which altered exonuclease activity but retained endonuclease activity. Since these mutations retained resolvase activity, we believe that the endonuclease activity of AN is an important resolvase and may be involved in herpesvirus replication.

4.5 References

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Chapter 5
Discussion

5.1 Conclusions and Future Directions

The herpes simplex type 1 alkaline nuclease (AN) was originally believed to be involved in DNA replication but was not found to be essential in the life cycle of the virus. However, AN is not one of the seven essential proteins needed for replication of viral DNA. The essential viral proteins are encoded by the UL5, UL8, UL9, UL29, UL30, UL42, and UL52 genes (Wu *et al.*, 1988). It has been speculated that AN may be involved in nucleic acid metabolism (Hoffmann and Cheng, 1978). HSV-1 encodes a number of metabolic proteins (uracil N-glycosylase, dUTPase, thymidine kinase (TK), ribonucleotide reductase) that are not essential for replication in rapidly growing cultured cells, but may play an important role in natural infection (Boehmer and Lehman, 1997). Strains of HSV-1 with mutations in uracil N-glycosylase, dUTPase, TK, or ribonucleotide reductase are less neurovirulent than wild-type virus in animal models (Boehmer and Lehman, 1997). The products from enzymatic digestion by AN are 5'-monophosphate nucleosides, which could be used as precursors for the triphosphate nucleosides necessary for DNA synthesis (Morrison and Keir, 1968; Hoffmann and Cheng, 1978). However, mutants in AN do not grow well in cultured cells as demonstrated by the null mutant AN-1 (Weller *et al.*, 1990). DNA synthesis and production of late proteins was sustained at wild-type levels, but the production of mature virions was severely restricted suggesting that there was a defect in processing or packaging of viral DNA (Weller *et al.*, 1990). More specifically, the defect appeared to be an inability of capsids to egress from the nucleus so that viral DNA containing C capsids are not found in the cytoplasm of the infected cell (Shao *et al.*, 1993).

Another clue about the role of AN was obtained from studies of the replicative intermediates formed during viral DNA replication. Although DNA replication is believed to proceed via a rolling circle mechanism (Jacob and Roizman, 1977; Becker *et al.*, 1978; Jacob *et al.*, 1979; Poffenberger and Roizman, 1985), simple linear head-to-tail concatemers are not formed in HSV-1 replication. Rather, the replicating DNA forms a complex mass of highly branched DNA (Shlomai *et al.*, 1976; Friedman *et al.*, 1977; Severini *et al.*, 1994, 1996). Therefore, debranching must occur before a viral genome can be packaged. This situation is similar to the replication in bacteriophage T4 in which recombination is used to initiate DNA synthesis (Mosig *et al.*, 1987, 1998). This form of replication results in the production of highly branched DNA (Luder and Mosig, 1982) which is debranched by the T4 gene 49 product, endonuclease VII, prior to packaging into preformed heads (Kemper and Janz, 1976; Kemper and Brown, 1976; Kemper and Garrabett, 1981). T4 endonuclease VII is a Holliday resolvase (Mizuuchi *et al.*, 1982; Lilley and Kemper, 1984; Kemper *et al.*, 1984) and mutants in gene 49 accumulate highly branched DNA that cannot be packaged (Kemper and Janz, 1976; Kemper and Brown, 1976). It was hypothesized that AN may play an analogous role such that the defect in AN-1 results in an inability to debranch replicating HSV-1 DNA into a proper linear form. This may result in abortive packaging (Shao *et al.*, 1993; Martinez *et al.*, 1996). In support of this idea, the replicative viral DNA intermediates were observed to be more branched in AN-1 infected cells than the DNA intermediates in wild-type HSV-1 infected cells (Martinez *et al.*, 1996). T7 endonuclease I, another Holliday resolvase, was shown to be able to remove the branches in replicative intermediates of HSV-1 DNA (Severini *et al.*, 1996). However, recently AN was shown to not have Holliday resolvase activity (Goldstein and Weller, 1998b). The objective of this thesis was to show that a Holliday resolvase activity is associated with HSV-1 infection and to pursue additional

studies to demonstrate resolvase activity in a viral encoded gene, most likely HSV-1 AN.

The first objective was to determine if HSV-1 infection resulted in the production of Holliday resolvase activity. This activity had not previously been described in HSV-1 infected cells. In chapter 2, I reported that a novel nuclease activity was produced in HSV-1 infected cells and absent in uninfected cells. This nuclease was partially purified and was found to possess both exonuclease and endonuclease activities that were more active at an alkaline pH. Also, it was determined that the exonuclease and endonuclease activities were detectable when $MgCl_2$ was used in reactions, but the exonuclease activity was inhibited when $MnCl_2$ was supplied as the divalent cation. This nuclease cleaved Holliday structures (cruciforms) created from extruded inverted repeats in supercoiled plasmids. This nuclease did not cleave the same plasmid when it was relaxed with topoisomerase I. The relaxed plasmid has the same sequence as the supercoiled plasmid, which suggested that a specific sequence was not recognized by the nuclease. It was suggested that this plasmid form (lineform) was not digested because it lacked the cruciform structure. To further characterize the nuclease activity, synthetic Holliday structures were synthesized from oligonucleotides. The cruciform used has a core of homology so that the branch point could migrate. Some resolvases, such as *E. coli* RuvC need a mobile cruciform for cleavage (Dunderdale *et al.*, 1991), whereas other resolvases, such as T4 endonuclease VII or T7 endonuclease I, have broader substrate specificity and can cleave both forms of cruciform equally well (Kemper *et al.*, 1984; Dickie *et al.*, 1987; Pottmeyer and Kemper, 1992). The products from cleavage of the synthetic cruciform by AN were identical to those produced by *E. coli* RuvC and T7 endonuclease I cleavage.

As mentioned above, it was speculated that AN may be involved in the resolution of branches. To determine if this Holliday resolvase activity was due to AN or due to another host cellular or viral protein, Holliday resolvase activity was examined in extracts prepared from wild-type HSV-1 infected, AN-1 infected, and mock infected cells The results of these studies showed that digestion of synthetic Holliday junctions was observed in extracts prepared form wild-type HSV-1, but absent in AN-1 and mock infected extracts. Therefore, Holliday resolvase activity was induced by HSV-1 infection of cells and AN was believed to play a role in this resolvase activity.

In chapter 3, HSV-1 AN was heterologously expressed in *E. coli* and purified to determine if AN has Holliday resolvase activity. In chapter 2, the nuclease was not highly purified and, therefore, it could not be determined if AN played a direct or indirect role in Holliday resolvase activity. The purified AN was shown to cleave a variety of supercoiled plasmids with different sizes of inverted repeat cloned into them and with different sequences. As well, AN was unable to digest supercoiled plasmids without inverted repeats. This suggested that AN does not recognize a particular sequence, but was structure specific. One of the experiments not done in chapter 2 was to map the exact site(s) of cleavage by AN. Previous characterization of AN had shown that it was able digest single and double stranded DNA with little specificity (Morrison and Keir, 1968; Hoffmann and Cheng, 1978, 1979; Strobel-Fidler and Francke, 1980; Hoffmann, 1981; Bronstein and Weber, 1996; Kehm *et al.*, 1998). Therefore, for a Holliday resolvase, we needed to show that cleavage occurred at the cruciform and that cleavage with symmetrical nicks across the base of the cruciform resulted in a linear product. First, linearization of a plasmid (supercoiled pSCB1a) by AN produced a linear product that had a hairpin at either end of the linear molecule. The formation of this product is consistent with Holliday resolvase cleavage (see fig

3.7). In another plasmid (pIR), cleavage occurred almost exclusively within the inverted repeat. Finer mapping by primer extension revealed that cleavage occurred at the base of the putative cruciform formed by extrusion of the inverted repeat. This specificity of cleavage was consistent with cleavage across the base of the cruciform and would be expected from a Holliday resolvase.

Previous work presented by Goldstein and Weller (1998b) showed that Holliday resolvase could not be demonstrated on synthetic Holliday junctions. Reactions with AN resulted in complete degradation. However, in chapter 3, a synthetic cruciform cleaved by purified AN formed linear duplex products. Taken together with the results from digestion of the plasmids, AN appears to have Holliday resolvase activity and that it might play a role in debranching the replicative intermediate prior to or during packaging.

In chapter 4, a series of point mutations in the UL12 gene that encodes AN were generated. Seven conserved motifs have been identified among the UL12 homologs within the herpesviuses (described in chapter 4 and Goldstein and Weller, 1998a). This conservation suggests that these motifs or domains are important for the function of AN. The targets chosen for mutagenesis were conserved acidic residues (aspartic acid and glutamic acid) in these motifs. Acidic residues have been identified as important for the catalytic activity by divalent metal cation-dependent nucleases (Suck, 1992; Gerit, 1993). The carboxylate groups of the acidic residues are important in bringing the divalent metal cations to the active site. Once properly coordinated, the metal cations can act as a proton acceptor for water to generate a hydroxide ion nucleophile for phosphodiester bond cleavage of the DNA substrate. Acidic residues have been identified as important for the catalytic activity of the Holliday resolvases *E. coli* RuvC, T4 endonuclease VII, and T7 endonuclease I (Saito *et al.*, 1995; Giraud-Panis and Lilley, 1996, White *et al.*, 1997; Parkinson *et al.*, 1999).

Five acidic residues were chosen for mutagenesis, 3 aspartic acids (E219, E364, E423) and 2 glutamic acids (D329, D340). For each site, the amino acid was substituted with alanine. In addition, for the aspartic acids, substitution with asparagine and glutamic acid was done and for the glutamic acids, substitution with glutamine and aspartic acid was done. These mutant nucleases were expressed in an *E. coli* expression system, purified and tested for exonuclease and endonuclease activity. The mutations made in E219, E364, and D340 resulted in total loss of activity, indicating that these residues are important for activity and that they may be important for the formation of the active site. Mutants in E423 displayed endonuclease and exonuclease activities identical to wild-type AN, indicating that this site is not involved in the active site. The mutants in D329 are the most interesting in that the mutations lost exonuclease activity, but retained endonuclease activity. The mutant D340E was previously generated and reported to also have lost exonuclease activity but retained endonuclease activity (Goldstein and Weller, 1998a). When I expressed this mutant, no activity was detected. One possibility for my inability to detect activity in D340E could be that D340E was improperly folded. Goldstein and Weller (1998a) produced D340E in baculovirus, whereas I produced D340E in *E. coli.* In baculovirus, nuclease expressed was soluble, whereas in *E. coli*, the nuclease was insoluble and must be denatured and refolded and this refolding step may have resulted in improper folding of the enzyme. Another hypothesis is that D340E may have endonuclease activity but no resolvase activity. In my thesis, assays for endonuclease activity were designed to check for resolvase activity (pH 7.5 with $MnCl_2$), whereas the assay for endonuclease activity by Goldstein and Weller (1998a) checked for digestion of pUC18 (pH 9 with $MgCl_2$). The latter hypothesis was favored because wild type AN, E423 mutants, and D329 mutants all had activity when refolded in the same manner as D340E. Consistent with this hypothesis is that Goldstein and Weller

(1998b) did not detect any activity on the synthetic substrates created from oligonucleotides used detect resolvase activity.

Since the D329 mutants retained endonuclease activity, these mutants were examined for Holliday resolvase activity. These mutants were able to cleave cruciform plasmids and the mapping of the cleavage sites by primer extension showed that the D329 mutants produced cleavages that mapped to the base of the cruciform. Also, these are the same sites of cleavage produced by T7 endonuclease I and wild-type AN. These results demonstrated that the endonuclease activity of AN was responsible for the Holliday resolvase activity.

The results demonstrating Holliday resolvase activity in AN were all done *in vitro*. It would be interesting to know if this function is an *in vivo* function. If it is, then the Holliday resolvase fits well with the model that it is involved in the debranching of replicative intermediates. This question was originally addressed by Goldstein and Weller (1998a) in which D340E was assessed for the ability to complement AN-1. Complementation was not observed, which led to the conclusion that exonuclease activity is the only activity needed for *in vivo* function. However, since D340E could not display Holliday resolvase activity (Goldstein and Weller, 1998b), it may explain the inability of the D340E mutant to complement AN-1. Also, in order to conclusively show that exonuclease activity is the only activity needed, a mutant that has lost endonuclease but retained exonuclease must be generated and be able to complement AN-1. I was unable to generate such a mutant and have not seen any reports of a mutant with this phenotype. Since the D329 mutants were demonstrated to have Holliday resolvase activity, it would be interesting to see if these mutants are able to complement AN-1. This could be done by transient expression in an non permissive AN-1 infected cell. In Goldstein and Weller (1998a), complementation was done by using amplicon vectors. Amplicon vectors are plasmids

which contain an HSV origin of replication and packaging sequence so that they can be replicated by HSV and packaged into defective viruses (Frenkel *et al.*, 1994). This method was reported to increase levels of transcomplementation because of the greater efficiency of infection over transfection (Berthomme *et al.*, 1996). Alternatively, the UL12 homolog of human cytomegalovirus, UL98, was able to complement a null mutant of AN when the UL98 gene was integrated into the genome of the null mutant (Gao *et al.*, 1998). The D329 mutants could be subcloned into amplicons or integrated into AN-1 to check for complementation.

It is possible that complementation might not occur. The discovery of Holliday resolvase activity does not preclude the idea that the exonuclease activity also has an important function and that both endonuclease/resolvase activity and exonuclease activities are necessary. The function of exonuclease would be easily addressed if a mutant that loses endonucleae activity but retains exonuclease activity could be produced. Speculation about the possible roles of the exonuclease is that it is involved in a repair process such as that proposed for *Neurospora crassa* endo-exonuclease (Goldstein and Weller, 1998b; Kehm *et al.*, 1998). The endo-exonuclease generates single-stranded DNA tails with 3'OH-termini by exonuclease digestion at double stranded DNA ends or nicks generated by endonucleolytic cleavage. These single-stranded tails are required for double-strand break repair of homologous DNA regions (Fraser *et al.*, 1989; Fraser, 1994). These gaps could result from Holliday endonuclease cleavage of branches which would generate sites where the exonuclease can act. Consistent with this idea is that Goldstein and Weller (1998b) showed that replicating DNA has no accessible double stranded ends but has nicks and gaps that AN can digest at.

Another proposed function for AN was that it may function to prevent 5' strand invasion (Goldstein and Weller, 1998b). In bacteriophage T4, 3' strand invasion leads

to the production of replication forks (Mosig, 1987, 1998). However, 5' end strand invasion would lead to non productive junctions. A similar situation is observed in *E. coli* by action of the RecJ exonuclease which degrades 5' invading strands (Corrette-Bennett and Lovett, 1995). Only 3' invading strands are productive for RecA mediated invasion of DNA during homologous recombination (Konforti and Davis, 1987). Currently, there is no evidence to support either hypothesis.

Recently, AN has shown to have homology with other nucleases (Goldstein and Weller, 1998a, Aravind *et al.*, 2000). These homologous proteins may provide insight into alternative functions of AN. The complete sequences of a number of baculoviruses have been determined and they all encode a homolog of AN that share motifs I,II, III, and VI with HSV-1 AN (Ayres *et al.*, 1994; Aherns *et al.*, 1997; Goldstein and Weller, 1998a; Hayakawa *et al.*, 1999; Ijkel *et al.*, 1999; Kuzio *et al.*, 1999). The nuclease from the *Autographa californica* multinuclepcapsid nucleopolyhedrovirus (AcMNPV) AN homolog, encoded by open reading frame 133, was purified and characterized (Li and Rohrmann, 2000). The AcMNPV AN was able to digest linear DNA at alkaline pH in $MgCl_2$. These are requirements similar to those required for HSV-1 AN. As well, mutations in motif II resulted in loss of activity (Li and Rohmann, 2000). However, the function of the baculovirus AN is not known.

HSV-1 AN has been reported to be a member of a superfamily of endonucleases called the LE superfamily typified by λ exonuclease (Aravind *et al.*, 2000) . λ exonuclease binds to free ends of dsDNA and degrades one strand 5'-3' (Kovall and Matthews, 1997). This creates 3' single-stranded overhangs that are used in DNA recombination by double-strand break repair (DSBR) and single-strand annealing (SSA) (Kowalczykowski *et al.*, 1994). In DSBR, the 3' ssDNA overhangs are bound by *E. coli* RecA and, subsequently, undergo strand exchange with a homologous piece of dsDNA (Kowalczykowski *et al.*, 1994). In SSA, the 3'

overhangs expose two homologous single-stranded regions of DNA that can anneal to form a recombinant DNA molecule (Kowalczykowski *et al.*, 1994). SSA is independent of RecA. The homology of λ exonuclease to HSV-1 AN may reveal an alternative function of AN other than Holliday resolvase activity. HSV-1 also has 5'-3' exonuclease activity (Knopf and Weisshart, 1990) and could generate ssDNA for either DSBR or SSA. With SSA, Holliday resolvase is not required. This presents an alternative model to the function of HSV-1 in initiation of recombination.

Another question that remains to be answered is the involvement of other proteins in debranching. Henderson *et al* (1998) reported that the N-terminal end of UL12 is extremely proline rich and proline rich regions have been implicated as motifs for protein- protein interactions. These regions may form a stable contact with other viral or cellular proteins. AN was shown to interact with ICP8 (Thomas *et al.*, 1992) and may interact with other replication proteins. Also, since AN has been shown to play a role in packaging (Weller *et al.*, 1990; Shao *et al.*, 1993; Martinez *et al.*, 1996), it is possible that there are interactions with other proteins involved in packaging. As well, it is possible that other proteins may modulate the activity of the nuclease to prevent total degradation of its replicating DNA. Fractionation of cell extracts from uninfected or AN-1 infected cells could be used to determine the effect of the extracts on AN activity.

Future studies could examine the contribution of residues other than acidic residues to catalysis or binding by AN. Henderson *et al.* (1998) demonstrated that a mutation at amino acid 150, which is a leucine and not within any of the conserved domains, completely abolished nuclease activity. The mutation G336A/S338A also had no activity. The contribution of these residues to activity is unknown. It is possible that these mutations could alter the binding of AN to the DNA substrate. This could be tested by, for example, electrophoretic mobility shift assays. In the

pseudorabies virus UL12 homolog, the histidine residue at amino acid 371, which corresponds to histidine 497 in HSV-1 UL12 in motif IV, was mutated to alanine and the mutant protein lost nuclease activity but retained DNA binding ability. In *E. coli* Ruv C (Ariyoshi *et al.*, 1994) and T7 endonuclease I (Parkinson *et al.*, 1999) basic residues such as lysine and arginine have been implicated as having roles in DNA binding. Conserved basic residues in the conserved motifs could be investigated by mutational analysis to see if they contribute to binding. Crystallization of AN would be very interesting. In my studies, *E. coli* expression produced a large amount of active nuclease and may be used as a source of the enzyme. Modeling of DNA substrates, or co-crystallization with DNA substrates, with AN may answer how specific residues contribute to activity.

5.2 References

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